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Massachusetts Institute of Technology
Woods Hole Oceanographic Institution

Joint Program in Oceanography/ Applied Ocean Science and Engineering

DOCTORAL DISSERTATION

Functional Genomics of a Non-Toxic *Alexandrium Lusitanicum* Culture

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Claudia A. Martins

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FUNCTIONAL GENOMICS OF A NON-TOXIC *ALEXANDRIUM LUSITANICUM* CULTURE

by
Claudia A. Martins

Lic. Biology
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FUNCTIONAL GENOMICS OF A NON-TOXIC CULTURE OF *ALEXANDRIUM LUSITANICUM*

by

CLAUDIA A. MARTINS

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ABSTRACT

Paralytic shellfish poisoning (PSP) is a human intoxication associated with the consumption of shellfish contaminated with a family of neurotoxins called saxitoxins. Many species in the dinoflagellate genus *Alexandrium* have been shown to produce these toxins. Here I report the first case of a culture of *Alexandrium* that has completely lost the ability to produce saxitoxins. The loss of toxicity was accompanied by a reduction in growth capability. A subculture of this isolate maintains the ability to produce toxins and to grow at rates and to cell abundances that were characteristic of the original *Alexandrium* culture. The growth and toxicity differences in the two isolates were demonstrated to be a property of the dinoflagellate itself and were not dependent on the different bacterial symbionts associated with each culture.

The pair of subcultures is a novel experimental system to study gene expression related to toxin production and growth in dinoflagellates. The products of gene expression were analyzed in the two subcultures of *Alexandrium* grown under the same conditions, but where toxicity and growth differ. At the metabolome level, compounds were identified that were unique to the non-toxic isolate; their emergence may be correlated to a disruption of the biosynthetic pathway for PSP toxins. These compounds share some characteristics and potential structural similarities with saxitoxins, though they are not any of the known toxin derivatives. Difference gel electrophoresis (DIGE) identified proteins differentially expressed between the two subcultures. Identification of some of these proteins was possible by searching the expressed sequence tag (EST) database for dinoflagellates. Proteins shown to be down-regulated in the non-toxic, slower growing subculture are all enzymes from the Calvin cycle, which may explain the limited growth of the non-toxic isolate. Other unknown, differentially expressed proteins may relate to the loss of toxicity, but their identity and function remain unresolved.

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CHAPTER 1

INTRODUCTION

A series of human syndromes have been linked to the consumption of shellfish or fish contaminated with toxins of microalgal origin. Paralytic shellfish poisoning (PSP) is arguably one of the most serious of these syndromes, as human intoxications can lead to death. The causative agent of PSP has been identified as a family of neurotoxins called saxitoxins. Dinoflagellates in the genera *Alexandrium, Pyrodinium*, and *Gymnodinium* (Cembella 1998) and a few freshwater cyanobacteria such as *Aphanizomenon*, *Anabaena* and *Lyngbia* (Humpage et al. 1994; Negri et al. 1997; Pereira et al. 2000) have been shown to produce these toxins. Bacteria have also been implicated in production of saxitoxin (Kodama et al., 1988) though evidence is less robust.

The negative economic, public health and ecosystem impacts of PSP outbreaks have been the common incentive for the many studies attempting to understand the mechanisms of saxitoxin production. Findings of this research have practical applications and will benefit the academic field, as well as commercial and governmental shellfish toxicity monitoring programs related to Public Health issues.

Though the chemical structure and activity of saxitoxins have been characterized (Shimizu 1993), little is known about their biosynthetic pathway or metabolic role in the dinoflagellate. The works of Shimizu (Shimizu 1996; Shimizu et al. 1985) are the only available studies that have provided information on the biosynthetic pathway of saxitoxin. By feeding labeled precursors to PSP toxin-producing organisms, Shimizu was able to propose a pathway that would lead to the synthesis of the perhydropurine ring of saxitoxin, as depicted in Figure 1 (Shimizu et al. 1990). As recognized by the author, the exact sequence of events, the number and identity of enzymes involved and, in some cases, the number of steps involved are at this point still a matter of speculation, and have yet to be confirmed in any subsequent studies (Cembella 1998).

The identification of the saxitoxin biosynthetic pathway is best studied at a molecular or genetic level. However, studies directed at the identification of genes related to toxin production have been limited by the lack of suitable experimental systems. The study of toxin production in dinoflagellates has long been restricted by the fact that it is not possible to completely suppress or elicit toxin production in toxic or non-toxic dinoflagellate cultures, respectively. To circumvent this, the choice of model organisms has been restricted to genetically related dinoflagellate strains that differ in the ability to produce toxins (eg. Taroncher-Oldenburg et al. 1997). Sequence dissimilarity between two strains, even closely related ones, however, is likely to interfere with studies looking into a few genes, as inter-specific variation is expected and does not necessarily reflect the differences in the ability to produce toxins (Scholin et al. 1994). Alternatively, dinoflagellate cells grown under N and P starvation, conditions that decrease and increase

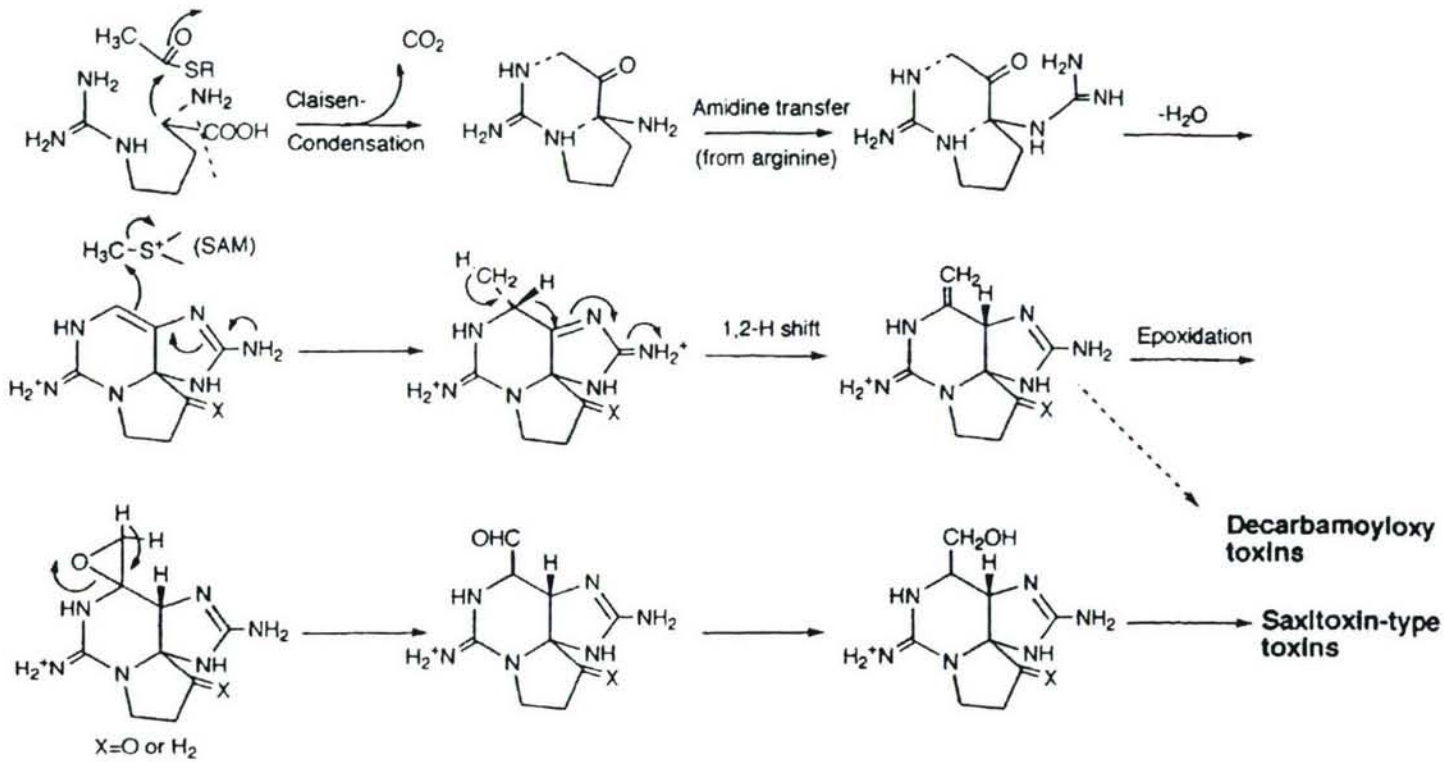

FIGURE 1-1. The PSP toxin biosynthetic pathway as proposed by Shimizu (1990). The pathway is based on the result of feeding experiments using labeled precursors.

toxin production, respectively, have been studied (D. Erdner, unpublished data). In this case, results will most likely reflect the incomplete suppression of toxin production under nitrate limited conditions. In addition, genes not necessarily involved in toxin production, but rather with nutrient stress, are also differentially expressed and will be identified (Erdner and Anderson, 2006). In fact, both approaches detailed above have failed to identify genes that can be definitely related to the production of PSP toxins.

The description of genes involved in toxin production, as well as enzymes and intermediary metabolites, has been recognized as being in part hindered by the inability to create and identify toxin-deficient mutant dinoflagellate cells (Plumley 2001). This difficulty is a result of both the biological characteristics of the dinoflagellate as well as technical constraints. The former includes high DNA/cell content of dinoflagellates (Rizzo 1987), the high number of copies of genes (Erdner and Anderson 2006; Le et al. 1997), limitations in culturing dinoflagellates on solid media, and the need to screen unrealistic numbers of clones in order to detect one mutant. Together these issues pose a logistical challenge where thousands of clonal cultures must be grown and analyzed for toxicity to detect one in which a toxin gene has been inactivated.

Experimental system

A potential step towards identifying naturally occurring mutants came in 2000, when routine analysis of dinoflagellate cultures maintained in the Laboratorio de Microbiologia Experimental (Portugal) culture collection revealed that a culture of *Alexandrium lusitanicum* was no longer producing PSP toxins (referred to as 18-1NT). Prior to this date, PSP toxins had been detected in every analysis, with a consistent toxin profile composed of gonyautoxins 1 to 4 (GTX1 – 4) (Table 1). A subculture of the same parental *A. lusitanicum* isolate, kept as a separate culture since 1992, however, maintained the same toxin profile and the same toxin content as previously reported (referred to as 18-1T). The lack of toxicity in a previously toxic *Alexandrium* culture was a surprising finding, as the ability to produce PSP toxins had been considered a constitutive characteristic. Indeed, prior studies had established that dinoflagellate cultures either have or do not have the ability to produce toxins, and never change between the two. Changes in toxin content and to a lesser extent the toxin profile of a culture do occur, typically following nutrient limitation (Anderson et al. 1990); however, the total loss of toxicity has never been described for *Alexandrium*.

In summary, a pair of subcultures of the same dinoflagellate culture was available, apparently differing in their ability to produce PSP toxins. The first step towards the experimental utilization of this system was to confirm that the loss of toxicity was real, and not an artifact of culture maintenance. Likely criticisms would be that the culture lost toxicity due to being switched or contaminated with another, non-toxic, culture. Furthermore, the total loss of toxicity also needed to be demonstrated, as low levels of toxicity, if present, would still constitute an argument for the constitutive nature of saxitoxin production.

Table 1-1. Source of the two subcultures of *A. lusitanicum* used in the study. Growth conditions of each subculture from 1962 to 2000 are summarized in the table.

YEAR	CONCLUSIONS	REFERENCE
1962	• 18-1 isolated from Lagoa de Obidos, Portugal • Maintained in ASP7 with addition of antibiotic mixture AM9 every 2 new transfer • Culture shown to be toxic by Mouse Bioassay	(Silva, 1962)
1987	• Toxin profile of 18-1 parental culture shown to be restricted to GTX1-4 by HPLC	(Cembella et al. 1987)
1992	• Subculture of 18-1 established and sent to Dr. Doucette's laboratory (USA) • Dr. Doucette changed the subculture to f/2 medium and stopped the antibiotic treatment	S. Franca, personal communication
1995	• Parental 18-1 culture shown to continue to produce GTX1-4 by HPLC	(Mascarenhas et al. 1995)
2000	• 18-1 parental culture seemed to be non-toxic (renamed 18-1NT) • 18-1 subculture in the USA remained toxic, producing GTX1-4 (renamed 18-1T)	(Pereira et al, 2000)
2000	• 18-1T and 18-1NT transferred to identical growth conditions. • Studies performed in both cultures kept in the same laboratory, in f/2 growth medium and without further antibiotic addition	

Bearing these arguments in mind, the hypothesis that the two *A. lusitanicum* were subcultures of the same isolate that differ only in their ability to produce saxitoxins was investigated. In other words, toxic and non-toxic cultures of *A. lusitanicum* were a spontaneously emerging mutant / wild-type pair. If this was established, the pair of cultures would represent a novel experimental system in which to study the mechanisms of toxin production. Because the loss of toxin production was subsequently shown to be accompanied by a reduction in growth, any studies performed would most likely also reveal the underlying processes involved in dinoflagellate growth.

The overarching hypothesis was that toxic and non-toxic *A. lusitanicum* strains have differences in their expressed genes that relate to the process of saxitoxin biosynthesis and growth capability. Being subcultures of the same original isolate, only differing in toxicity and growth capability, the gene expression patterns of both should be highly shared, except for those related to the different phenotype. It was also hypothesized that the toxicity and growth differences could relate to the associated bacterial communities in these two cultures.

Bacterial influence on toxicity and growth

One main difference between the maintenance conditions of the two *A. lusitanicum* subcultures involved the regular use of antibiotics in the non-toxic clone's growth medium. The mixture contained several antibiotics that could have affected specific bacteria, potentially altering the bacterial assemblage of 18-1NT. The change of bacterial community was thus suspected as a factor influencing the changes in growth and toxin production in the experimental system. Silva (1962) first proposed a role of bacteria in dinoflagellate saxitoxin production. This hypothesis has been further supported by the body of scientific work detailing the effects of bacteria in some algal cultures (Gallacher and Smith 1999). In particular, Sousa Silva (1990) reported that toxin production can be elicited in a non-toxic dinoflagellate by the addition of bacteria from a toxic culture. Furthermore, removing bacteria from dinoflagellate cultures has been shown to reduce the toxin content dramatically (Doucette and Powell 1998; Uribe and Espejo 2003), though a total loss of toxin production has never been reported. Therefore, it was conceivable that the loss of toxin production could be linked to the associated bacteria. This hypothesis was investigated by assessing the effect of different bacterial assemblages on the toxicity and growth of the non-toxic subculture.

Independent of the effect of bacteria on dinoflagellate toxin production, the autonomous production of saxitoxin by bacteria is more or less discounted (Baker et al. 2003; Martins et al. 2003). Furthermore, axenic cultures of dinoflagellates have also been shown to produce saxitoxins, though sometimes at a lower level than their xenic counterpart. Therefore, this thesis is based on the assumption that dinoflagellates are directly producing saxitoxins, independently of the initiating factor. In other words, bacteria may promote the production of toxins, but the metabolic or synthetic processes occur solely in the dinoflagellate cells.

Methodology

The choice of methodology for this study was based on both technical and biological considerations, in an attempt to maximize the amount of information that could be obtained from the experimental system. The unique characteristics of dinoflagellates greatly influence the choice of methodology to study differentially expressed genes in the two *A. lusitanicum* subcultures. For one, dinoflagellates possess disproportionately large genomes, with a DNA content range from 3 to 250 pg•cell^{-1} (Spector 1984). This makes it intractable to use simple genomic hybridizations like Southern blots and impractical to construct comprehensive genomic libraries.

On the other hand, post-transcriptional gene regulation is widely used by eukaryotes, especially for compounds transcribed from more than one gene unit (Lodish et al. 2000). Post-transcriptional gene regulation has been demonstrated to occur in dinoflagellates, for example in some genes of interest such as *lbp* (Morse et al. 1996) and GAPDH (Fagan et al. 1999). These types of regulatory mechanisms imply that the mRNA analysis of a dinoflagellate cell may not reflect the proteins, and consequently the phenotype, expressed by a cell at any time.

Therefore, the work reported in this thesis focused on the final products of gene expression, namely, the proteome and the metabolome, rather than focusing on genome and transcriptome characterization of the two *Alexandrium* subcultures.

Proteomics

The study of the proteome, rather than the transcriptome, would have a further advantage in cases where a point mutation was responsible for the loss of saxitoxin production. A point mutation would not necessarily produce a substantially different mRNA, but there is a higher chance that different or non-functional proteins would be produced. In the case of aflatoxin- vs non-aflatoxin-producing species of *Aspergillus*, for example, the inability to produce toxins seems to be related to a single nucleotide change that results in the loss of the final 65 aa of the expressed protein (Chang 2004). Therefore, protein profiles obtained for the two *A. lusitanicum* cultures will most likely reflect transcriptional, post- transcriptional and translational modifications related to the loss of toxin production as well as the observed differences in growth capability.

Dinoflagellate proteomics is still a new field, with a very small number of expressed proteins described (Chan et al. 2002). To our knowledge only one laboratory has successfully applied such methodology to the study of this group of algae. Recently, (Chan et al. 2004) used two-dimensional polyacrylamide gel electrophoresis (2-D) technology to distinguish between HAB species and to identify biomarkers of toxicity. The results from their 2-D electrophoresis efforts created both an initial methodology and an initial database of expressed proteins for dinoflagellates that will greatly benefit further studies.

Metabolomics

All information expressed by a genome ultimately leads to the production of a specific array of metabolites. The field of metabolomics, i.e., the description of all compounds produced by an organism, has recently gained attention due to the potential for discovery of new metabolites of commercial interest and to assign biochemical pathways to an organism (Fridman and Pichersky 2005).

Rather than attempting to characterize the total suite of metabolites produced by each *A. lusitanicum* culture, this study focused on compounds that could be detected by high performance liquid chromatography (HPLC), with the mobile and oxidant phases optimized to detect saxitoxin and its derivatives. This approach was based on the hypothesis that an interruption of the biochemical pathway leading to toxin production occurred in the non-toxic *A. lusitanicum* culture, causing precursors, metabolites or degradation products to accumulate in the cells. These compounds would most likely share some characteristics with the saxitoxin molecule and could possibly be detected in HPLC analysis.

CONCLUSIONS

A unique experimental system has potentially been identified for the study of toxin production and growth in dinoflagellates. The mechanisms responsible for the observed phenotypical changes were investigated by analyzing the *A. lusitanicum* culture's functional genomics, in particular, the proteome and the metabolome of the organism.

The main focus of this study was on the products of gene expression that are potentially related to toxin production within the two *A. lusitanicum* clones. Despite the emphasis on identifying proteins and metabolites involved in saxitoxin biosynthesis, the application of these functional genomics techniques is likely to provide valuable information on many other biological processes occurring in the cell. Dinoflagellates are a relatively under-studied experimental system, and the use of this methodology has the potential to uncover novel genes and add to the data base of genetic information for these organisms.

REFERENCES

Anderson, D. M., D. M. Kulis, J. J. Sullivan, S. Hall, and C. Lee. 1990. Dynamics and physiology of saxitoxin production by the dinoflagellates *Alexandrium* spp. Marine biology **104:** 511-524.

Baker, T. R., G. J. Doucette, C. Powell, G. L. Boyer, and F. G. Plumley. 2003. GTX4 imposters: characterization of fluorescent compounds synthesized by *Pseudomonas stutzeri* SF / PS and *Pseudomonas* / *Alteromonas* PTB-1, symbionts of saxitoxin-producing *Alexandrium* spp. Toxicon **41:** 339-347.

Cembella, A. D. 1998. Ecophysiology and metabolism of paralytic shellfish toxins in marine microalgae, p. 381-403. *In* D. M. Anderson, A. D. Cembella and G. M.

Hallegraeff [eds.], Physiological Ecology of Harmful Algal Blooms. NATO ASI Series. Springer-Verlag.

Cembella, A. D., J. J. Sullivan, G. L. Boyer, F. J. R. Taylor, and R. J. Andersen. 1987. Variation in paralytic shellfish toxin composition within the *Protogonyaulax tamarensis/catenella* species complex; red tide dinoflagellates. Biochemical Systematics and Ecology **15:** 171-186.

Chan, L. L., I. J. Hodgkiss, S. Lu, and S. C. Lo. 2004. Use of two-dimensional gel electrophoresis proteome reference maps of dinoflagellates for species recognition of causative agents of harmful algal blooms. Proteomics **4:** 180-192.

Chan, L. L., S. C. Lo, and I. J. Hodgkiss. 2002. Proteomic study of a model causative agent of harmful red tide, *Prorocentrum triestinum* I: Optimization of sample preparation methodologies for analyzing with two-dimensional electrophoresis. Proteomics **2:** 1169-1186.

Chang, P. 2004. Lack of interaction between AFLR and AFLJ contributes to nonaflatoxigenicity of *Aspergillus sojae*. Journal of Biotechnology **107:** 245-253.

Doucette, G. J., and C. Powell. 1998. Algal-bacteria interactions: can they determine the PSP-related toxicity of dinoflagellates, p. 406-409. *In* B. Reguera, Blanco, J., Fernandez, M.L. and Wyatt, T. [ed.], Harmful algae. Xunta de Galicia and IOC.

Erdner, D., and D. M. Anderson. 2006. Global transcriptional profiling of the toxic dinoflagellate *Alexandrium fundyense* using Massively Parallel Signature sequencing. BMC Genomics **7:** 88.

Fagan, T. F., D. Morse, and J. W. Hastings. 1999. Circadian synthesis of a nuclear-encoded chloroplast glyceraldehyde-3-phosphate dehydrogenase in the dinoflagellate *Gonyaulax polyedra* is translationally controlled. Biochemistry **38:** 7689-7695.

Fridman, E., and E. Pichersky. 2005. Metabolomics, genomics, proteomics and the identification of enzymes and their substrates and products. Current Opinion in Plant Biology **8:** 242-248.

Gallacher, S., and E. Smith. 1999. Bacteria and Paralytic Shellfish Toxins. Protist **150:** 245-255.

Humpage, A. R. and others 1994. Paralytic shellfish poisons from Australian cyanobacterial blooms. Australian Journal of Marine and Freshwater Research **45:** 761-771.

Le, Q. H., P. Markovic, J. W. Hastings, R. V. M. Jovine, and D. Morse. 1997. Structure and organization of the peridinin-chlorophyll a-binding protein gene in *Gonyaulax polyedra*. Molecular and General Genetics **255:** 595-604.

Lodish, H., A. Berk, L. S. Zipursky, P. Matsudaira, D. Baltimore, and J. Darnell. 2000. Molecular cell biology, 4th ed. WH Freeman and Company.

Martins, C. A., P. Alvito, M. J. Tavares, P. Pereira, G. Doucette, and S. Franca. 2003. Reevaluation of production of paralytic shellfish toxin by bacteria associated with dinoflagellates of the Portuguese coast. Applied and Environmental Microbiology **69:** 5693-5698.

Mascarenhas, V., P. Alvito, S. Franca, I. Sousa, J. A. Rodriguez-Vasquez, and A. G. Martinez. 1995. The dinoflagellate *Alexandrium lusitanicum* isolated from the coast of Portugal: observations on toxicity and ultrastructure during growth phases, p. 71-76. *In* P. Lassus, G. Arzul, E. Erard-Le Denn, P. Gentien and C.

Marcaillou-Le Baut [eds.], Harmful Marine Algal Blooms/Proliferation d'Algues Marines Nuisibles. Proceedings of the Sixth International Conference on Toxic Marine Phytoplankton, Nantes (France), Oct 1993. Lavoisier.

Morse, D., P. Markovic, and T. Roenneberg. 1996. Several clocks may simplify the circadian system of *Gonyaulax*. Brazilian Journal of Medical and Biological Research **29:** 101-103.

Negri, A. P., G. J. Jones, S. I. Blackburn, Y. Oshima, and H. Onodera. 1997. Effect of culture and bloom development and of sample storage on paralytic shellfish poisons in the cyanobacterium *Anabaena circinalis*. Journal of Phycology **33:** 26-35.

Pereira, P. and others 2000. Paralytic shellfish toxins in the freshwater cyanobacterium *Aphanizomenon flos-aquae*, isolated from Montargil reservoir, Portugal. Toxicon **38:** 1689-1702.

Plumley, F. G. 2001. Purification of an enzyme involved in saxitoxin synthesis. Journal of Phycology **37:** 926-931.

Rizzo, P. 1987. Biochemistry of the dinoflagellate nucleus, p. 143-173. *In* F. Taylor [ed.], The biology of dinoflagellates. Blackwell Scientific.

Scholin, C. A., M. Herzog, M. Sogin, and D. M. Anderson. 1994. Identification of group- and strain-specific genetic markers for globally distributed *Alexandrium* (Dinophyceae). 2. Sequence analysis of a fragment of the LSU rRNA gene. Journal of Phycology **30:** 999-1011.

Shimizu, Y. 1996. Microalgal metabolites: A new perspective. Annual Review of Microbiology [Annu. Rev. Microbiol.] **50:** 431-465.

Shimizu, Y., S. Gupta, M. Norte, A. Hori, A. Genenah, and M. Kobayashi. 1985. Biosynthesis of paralytic shellfish toxins, p. 271-274. *In* D. M. Anderson, A. W. White and D. G. Baden [eds.], Toxic Dinoflagellates. Proceedings of the Third International Conference on Toxic Dinoflagellates, St. Andrews, N.B. (Canada), 8-12 Jun 1985. Elsevier.

Shimizu, Y., S. Gupta, and A. V. K. Prasad. 1990. Biosynthesis of dinoflagellate toxins, p. 62-73. *In* E. Graneli, B. Sundstroem, L. Edler and D. M. Anderson [eds.], Toxic Marine Phytoplankton. Proceedings of the Fourth International Conference on Toxic Marine Phytoplankton, Lund (Sweden), 26-30 Jun 1989.

Silva, E. S. 1962. Some observations on marine dinoflagellate cultures. I. *Prorocentrum micans* Ehr., *Gymnodinium spinifera* (Clap. and Lach.) dies., *Gonyaulax tamarensis* Leb., and *Peridinium trochoideum* (Stein) Lemm. Notas e estudos do Inst Biol Mar **26:** 1-21.

Sousa Silva, E. 1990. Intracellular bacteria: the origin of dinoflagellate toxicity. Journal of Environmental Pathology, Toxicology, and Oncology **10:** 124-128.

Spector, D. L. 1984. Dinoflagellate Nuclei, p. 107-147. *In* D. L. Spector [ed.], Dinoflagellates. Academic Press, Inc.

Uribe, P., and R. T. Espejo. 2003. Effect of associated bacteria on the growth and toxicity of *Alexandrium catenella*. Applied and Environmental Microbiology **69:** 659-662.

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The loss of PSP toxin production in a formerly toxic *Alexandrium lusitanicum* clone

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Abstract

Toxin production has always been considered a constitutive characteristic of dinoflagellates in the genus *Alexandrium*. Here we demonstrate that saxitoxin production can be lost by an *Alexandrium* species during routine culture maintenance. This is the first report of any marine saxitoxin-producing alga ever to have completely lost the ability to produce toxins. A clonal toxic isolate of *Alexandrium lusitanicum* from Portugal has been maintained in culture since 1962. In 1992, a subculture was established and sent to a different laboratory. Recent comparisons of the parental strain and the subculture revealed that the former had lost its toxicity, whereas the latter still produces saxitoxins. This loss of toxicity was confirmed by three independent toxin detection methods: mouse bioassay, mouse neuroblastoma assay and HPLC. Sequence analyses of different rRNA domains demonstrated that the toxic and non-toxic cultures are genetically identical for those markers. Morphological analysis showed that both cultures have the same plate tabulation and are *A. lusitanicum*. These results strongly argue that the loss of toxicity is not a result of a culturing artifact or mistake, such as mislabeling or contamination. The clonal cultures also show a significant difference in growth. Possible explanations for the change include genetic mutations or the effects of prolonged treatment of the non-toxic culture with antibiotics.

Keywords: *Alexandrium*; Loss of toxin production; Paralytic shellfish poisoning; Saxitoxin

1. Introduction

Species within the dinoflagellate genus *Alexandrium* have been associated with paralytic shellfish poisoning (PSP) episodes in coastal waters throughout the world, from boreal to tropical latitudes. The saxitoxins, a family of potent neurotoxins, are produced by some of these species in varying amounts and combinations, leading to highly variable intrinsic potencies (Cembella, 1998). To date more than 20 saxitoxin derivatives that differ considerably in toxicity have been identified (Cembella, 1998; Gallacher and Smith, 1999), and numerous *Alexandrium* isolates have been characterized with respect to their toxin profile or composition (i.e. the suite of toxins produced).

Under normal growth conditions, a species or strain produces a consistent or characteristic toxin profile and toxin content (total toxicity) (Hall, 1982; Cembella et al., 1987; Anderson, 1990; Anderson et al., 1994). However, these and other studies have documented variation in toxin content (Anderson et al., 1990a; Anderson, 1990; Mascarenhas et al., 1995) and toxin composition (Anderson et al., 1990b; Franco et al., 1995), typically following sustained nutrient deprivation and adaptation. These variations reflect differences in a strain's growth conditions, such as nitrate and phosphate concentrations (Anderson et al., 1990c) or salinity (Flynn et al., 1996). Regardless of the variations in toxin content and composition in a strain, toxin production (or the lack thereof) is considered a highly stable

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characteristic of *Alexandrium* strains, i.e. isolates always produce some toxin, or none at all, but never switch between these two conditions. Although some strains kept in culture are reported to suffer reduction in toxin content (Hansen et al., 2003) the total loss of the ability to produce saxitoxins has never been observed to date.

The constitutive nature of toxin production seems to be true for other toxic marine algae, although there are a few exceptions. There are, for example, no reports of the total loss of brevetoxin toxicity in *Karenia brevis* cultures, but one strain apparently lost its ability to produce one of the brevetoxin derivatives while in culture (R. Pierce pers. comm.). For the diatom *Pseudo-nitzschia multiseries*, there are times during the growth cycle (exponential growth), when a strain will produce no domoic acid, but that same strain will produce toxin when growth slows or ceases in stationary phase (Bates, 1998). In this instance, the capability to produce toxin has not been permanently lost-toxin synthesis has simply been turned off during a particular growth phase.

One protist that can potentially alternate between toxic and non-toxic forms is *Pfiesteria piscicida*. For example, when toxic zoospores of *P. piscicida* are grown in culture with algae as food, they gradually lose their toxicity (Burkholder and Glasgow, 1997). Springer (2000) reports that some zoospores have residual levels of toxicity, and that full toxicity can be re-established by growing the now non-toxic zoospores on fish prey. Other workers report cases in which the non-toxic zoospores remain uninducible (Stoecker et al., 2002). These results suggest that toxin production can be lost in this species, but until the *Pfiesteria* toxin is chemically identified and analyzed, it is premature to state that toxin production is completely lost.

A culture of *Prorocentrum minimum* may represent another exception to the tendency towards constitutive toxin production. In a study by Sousa Silva (1990), bacteria previously isolated from a toxic *Gonyaulax tamarensis* (= *Alexandrium tamarense*) culture were added to a non-toxic clone of *P. minimum*. The inoculated clone showed some toxicity (as evidenced by mouse bioassay) while the controls remained non-toxic. Similar results were obtained for two clones of *Gyrodinium instriatum*, leading Sousa Silva to conclude that toxicity could be elicited in clones by adding the *A. tamarense* bacteria. Although this seems to be a case of de novo toxin production, the type of toxin produced was not defined and may not have even been saxitoxin. Furthermore, these results have not been successfully repeated by others.

Considering all available data, the ability to produce PSP toxins has heretofore been demonstrated to be a constitutive, highly stable characteristic in marine algae in general, and, with no exception, in the genus *Alexandrium*. For the first time, here we report the total loss of toxicity of a clone of *Alexandrium lusitanicum*.

2. Materials and methods

2.1. Culture history

The original culture of *A. lusitanicum* was isolated in 1962 from a bloom in Lagoa de Obidos, Portugal and given the strain designation 18-1. That strain has been maintained at the Laboratório de Microbiologia e Ecotoxicologia in Lisbon ever since, using Provasoli's ASP7 medium with bimonthly addition of AM9 antibiotic mixture every two new transfers (Silva and Sousa, 1981). AM9 is a mixture of polymixin B sulfate, dihydrostreptomycin, tetracycline, chloramphenicol, penicillin G and neomycin. In 1992, a subculture was established and sent to Dr Greg Doucette's Laboratory. The culture was kept in f/2 media (enriched Vero Beach, Florida seawater) with no further antibiotic treatment. Salinity was kept at 30 psu, similar to the ASP7 medium. We refer to this subculture as 18-1T since it retains all of the characteristics of the parent culture isolated in 1962. At some unknown point between 1995 (Franca et al., 1995; Mascarenhas et al., 1995) and 2000 (Pereira et al., 2000), the 18-1 culture maintained at the Laboratório de Microbiologia e Ecotoxicologia became non-toxic. For discussion purposes, this culture is now referred to as 18-1NT to distinguish it from the parent culture 18-1 and the other subculture 18-1T.

2.2. Growth

Both 18-1T and 18-1NT were grown in identical conditions for at least 2 years before performing the experiments reported here. Cultures were maintained in f/2 medium (Guillard and Ryther, 1962) at 20 ± 1 °C on a 16:8 h L:D cycle and transferred in mid-exponential phase. The growth of each culture was followed by periodic cell counts with a Sedgwick-Rafter counting chamber. All counts were made in triplicate. Additionally, a second set of cultures was established simultaneously in f/2 and in ASP7 media (Provasoli, 1963), which is an artificial seawater-based culture medium. Growth in ASP7 was monitored following the same conditions and quantification methods described for f/2. Separate cultures were grown for toxin analysis, morphology and sequencing (see below).

2.3. Morphology

The plate morphology of each of the two subcultures was studied by calcofluor staining (Fritz and Triemer, 1985). Three ml of mid-exponential phase cultures were fixed in 5% v/v formalin, treated with calcofluor and observed under an epifluorescence microscope.

2.4. Ribossomal gene sequencing

Cells were collected by filtration from mid-exponential culture and frozen in liquid nitrogen prior to DNA

extraction. A modified 3% hexadecyltrimethyl-ammonium bromide (CTAB) protocol (Doyle and Doyle, 1990) for DNA extraction was used following mechanical cell disruption in a bead beater (Biospect Products). Two regions of the large subunit (Lsu) ribosomal RNA (rRNA): D1/D2 and D9/D10 were amplified from the extracted genomic DNA. Primers used for D1/D2 fragment were those published by Scholin et al. (1994). The region including the D8, D9 and D10 hypervariable domain was amplified using the FD8 and RB primers (Chinain et al., 1998). In addition to the large subunit amplifications, the ITS1, 5.8S and ITS2 regions were also amplified by PCR using the ITS1 and ITS 4 universal primers described in D'Onofrio et al. (1999). The same PCR conditions were used for all amplifications, following the conditions described in Scholin and Anderson (1994).

The amplified regions were sequenced on an ABI automatic sequencer, following the reaction protocols described by the manufacturer. Reactions were completed with the PCR primers used. Sequences thus obtained were compared to each other and to a selected culture of *A. minutum* (AMD21), kindly provided by Santiago Fraga (Instituto Español de Oceanografia).

2.5. Toxin analysis

Mouse bioassay. Toxicity of cell extracts of both 18-1T and 18-1NT was first determined by mouse bioassay. Toxin extraction was performed from cell pellets from mid-exponential cultures, following the procedure as described in AOAC (1980). To ensure cell disruption, previous to boiling, samples in 10 ml of 0.1N HCl were sonicated. Extracts were maintained at pH3. Charles River test mice were injected intra-peritoneally with 1 ml of each acidic extract. All injections were performed in triplicate and time of death recorded. Two mice were also injected with 1 ml of 0.1N HCl as a control.

HPLC. Cultures grown in 25 ml f/2 medium were collected via centrifugation (5000*g*, 5 min, 23 °C) for toxin content and toxin profile measurement by HPLC. One millilitre of 0.05N acetic acid was added to the cell pellet and 3, 25 μl aliquots were removed from the mixed sample and preserved in separate 1 ml volumes of filtered seawater containing Utermöhl's solution (Utermöhl, 1958) for cell counts. The acetic acid cell slurry was then disrupted by sonication (10 W, 20 s.) in an ice bath. The samples were stored at −20 °C prior to analysis. A modification of the post-column oxidation method of Oshima (1995) was performed to quantify 14 saxitoxin derivatives as described in Anderson et al. (1994). Four mixtures of PSP toxin standards kindly provided by Prof. Oshima (Tohoku University, Sendai, Japan), were used for identification and quantification purposes. Toxicities of the derivatives (in fg STX equivalents $cell^{-1}$) were calculated from the molar composition data using individual potencies in mouse units per μmol^{-1} (Oshima, 1995): C1—15; C2—239; C3—32; C4—143; GTX1—2468; GTX2—892; GTX3—1584; GTX4—1803; GTX5—160; dcGTX2—382; dcGTX3—935; NEO—2295; dcSTX—1274; STX—2483.

Cells pellets were also collected from mid-exponential cultures grown in the two different growth media, f/2 and ASP7. To insure maximum cell number was not a factor in toxin detection, triplicates from each set of experiments were pooled together and processed as a single sample. Thus, four pellets were analyzed: the subculture grown in f/2 and ASP7 media and the parental culture grown in f/2 and ASP7 media. Toxin extraction and analysis were performed following the same experimental procedures described above.

Mouse neuroblastoma assay (MNA). For toxin content estimation by MNA, 250 ml of mid-exponential phase cultures were centrifuged. Toxin extraction was performed by mechanical disruption in a bead beater with glass beads in 0.05N acetic acid. A cell extract was also obtained from a culture of *Scrippsiella*, grown in the same conditions, for use as a negative control. All pellets were obtained in triplicate.

The MNA was carried out according to the method described by Gallacher and Birkbeck (1992). Cell extracts were assayed using the following dilutions in RPMI assay medium: 1/8, 1/16, 1/32, 1/64. Measured sodium channel blocking (SCB) activity was compared to the same dilutions of the *Scrippsiella* extract. This negative control was used to detect the effect of the cellular extract (no toxin) on neuroblastoma cells and its SCB activity per se. The combination of ouabain and veratridine that killed 80% of the neuroblastoma cells was previously determined by titration and chosen for routine detection of saxitoxin-like activity (typical values used were around 0.2 mM ouabain and 0.05 mM veratridine). SCB activity was compared to a STX dose-response curve using a certified reference standard (National Research Council, Halifax, Canada). Cell pellets from cultures grown simultaneously in f/2 and ASP7 media were also analyzed by MNA.

3. Results

3.1. Morphology

Examination of the thecal plates of the 18-1T and 18-1NT cells by calcofluor staining revealed no morphological differences between the two strains. Both possessed the plate formula characteristic of the Genus *Alexandrium* (Po, 4′, 6″, 5‴ 2⁗, 6c and 9–10 s, with the 1′ linked to the Po) (Fig. 1). The characteristics described by Balech (1995) that distinguish *A. lusitanicum* from the closely similar *A. minutum* (namely the narrow 6″ precingular plate and Type B form of the 2⁗ plate) were also observed in both strains.

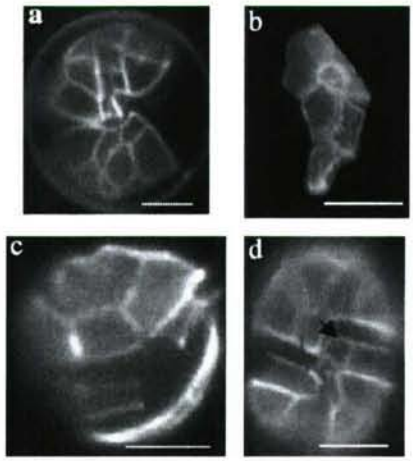

Fig. 1. Morphology of *Alexandrium lusitanicum* strains 18-1NT top, and 18-1T, bottom. Scale bar: approximately 12.5 μm. (a) General view of 18-1NT. (b) detail of $1'$, $4'$ plates and ventral pore. (c) Back, lower half view of 18-1T. Visible are the $2'''$, $2'''$, $3'''$, $4'''$ plates. (d) Details of the anterior sulcal plate (indicated by the arrow).

Table 1
GenBank accession numbers of strains sequenced

Strain/rDNA	D1/D2	D8/D10	ITS
18-1T	AY455827	AY455824	AY455826
18-1NT	AY455828	AY455823	AY455825

3.2. Ribosomal RNA gene sequencing

PCR amplification of the D1/D2 region of the Lsu rRNA gene produced fragments of approximately 665 bp. The 18-1T and 18-1NT strains had identical nucleotide sequences that differed from *A. minutum* by a single base (Table 1, Fig. 2). Similarly, no genetic variation between the two *A. lusitanicum* cultures was detected with the D8/D10 primers. In contrast to the results for the D1/D2 primers, the nucleotide sequences of the 720 bp fragment of all three cultures tested were identical, including that for *A. minutum*. Similar results were obtained for the whole ITS1, 5.8S and ITS2 region. Again, no nucleotide variations were detected between *A. lusitanicum* 18-1T, 18-1NT and *A. minutum*.

3.3. Growth

Significant differences in growth were observed between the two strains when grown in f/2 medium (Fig. 3A). 18-1NT was consistently slow growing and attained a lower maximum cell density. In f/2 medium, the maximum cell density reached was 20,124 ± 1356 cell ml^{-1} for 18-1NT versus 33,167 ± 2420 cell ml^{-1} for 18-1T, while calculated growth rates were 0.14 d^{-1} for 18-1NT and 0.19 d^{-1} for the toxic 18-1T.

Growth of the same two cultures was also measured in ASP7 media, with the same general results as for f/2 medium (Fig. 3B). The toxic 18-1T again showed a higher growth rate and attained a higher maximum cell yield. For both cultures, however, cell yield and growth rate were consistently lower than was the case in f/2 media. In ASP7, cell yield of 18-1T did not exceed 26,080 ± 4689 cell ml^{-1}. A similar pattern was observed for the 18-1NT (20, 130 ± 1356 cell ml^{-1} in f/2 vs. 13,900 ± 4291 cell ml^{-1} in ASP7).

3.4. Toxicity

Mouse bioassay. Cell pellets of at least 22×10^6 cells were used for this assay. The mice injected with 0.1N HCl or with the 18-1NT extract had a similar negative response. All mice survived the injection and did not show any unusual symptoms or behavior. Mice injected with the 18-1T extract died within 8 min and the median toxicity calculated was 4.1 MU or 352.8 fg STX.$cell^{-1}$.

HPLC. Analysis of the cell pellet extracts for the 18-1T and 18-1NT strains of *A. lusitanicum* using HPLC were remarkably different from each other. The chromatograms obtained for 18-1T showed a toxin profile consisting of mainly GTX 1, 4, with lower quantities of GTX 2, 3 (Fig. 4). No compounds with retention times comparable to the standards were detected in the STX and N-sulfocarbamoyl isocratic runs (Fig. 5). Total toxin content of the 18-1T extract was calculated to be 8.4 ± 0.4 fmol $cell^{-1}$ in f/2, or 3784.6 + 150.8 fg STX equivalents.$cell^{-1}$.

```
18-1-NT  ATATGGTTGATGTGGGTGCGATGGTTCTTACCTTGAATGTCAGCTTCTATTTCTGCAAAT
AMD21    ATATGGTTGATGTGGGTGCGATGGTTCTTACCTTGAATGTCAGCTTCTATTTCTGCAAAT
18-1-T   ATATGGTTGATGTGGGTGCGATGGTTCTTACCTTGAATGTCAGCTTCTATTTCTGCAAAT

18-1-NT  CATTACCCTTGCACATGAATGGTAATTTGCCTGCGGGTATTGGAATGCATGTGTTTGCAA
AMD21    CATTACCCTTGCACATGAATGGTAACTTGCCTGCGGGTATTGGAATGCATGTGTTTGCAA
18-1-T   CATTACCCTTGCACATGAATGGTAATTTGCCTGCGGGTATTGGAATGCATGTGTTTGCAA

18-1-NT  TGATTTGTGATTTGACGCATGTGTTTGGTGAAATTTGTATATGCTCTTTTGTCGAAGGGG
AMD21    TGATTTGTGATTTGACGCATGTGTTTGGTGAAATTTGTATATGCTCTTTTGTCGAAGGGG
18-1-T   TGATTTGTGATTTGACGCATGTGTTTGGTGAAATTTGTATATGCTCTTTTGTCGAAGGGG
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Fig. 2. Partial sequence of isolates 18-1T, AMD21 and 18-1NT cultures for the D1/D2 region of the Lsu rRNA. Bases 420–600 are represented. The base pair difference between 18-1 and AMD21 is highlighted.

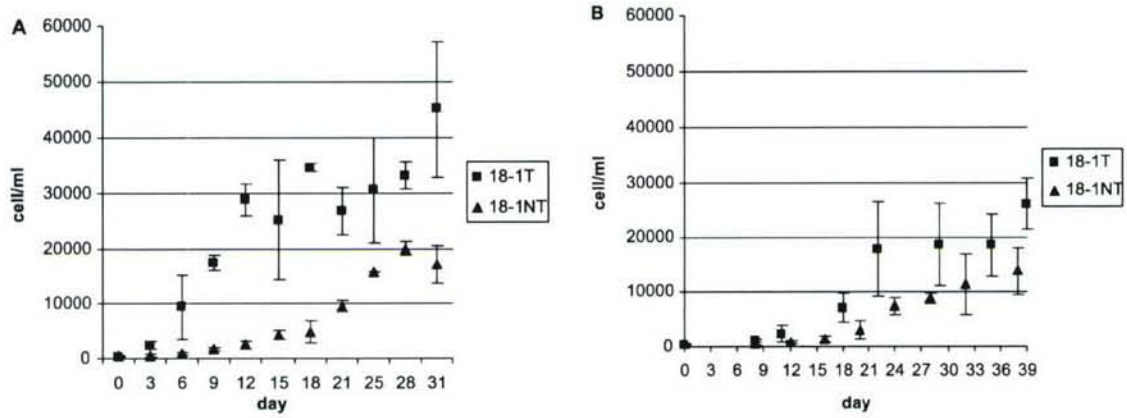

Fig. 3. Comparison of cell numbers of the non-toxic 18-1NT and toxic 18-1T isolates grown in f/2 (A) and ASP7 (B) media. Standard deviations are shown for each count ($n = 3$).

In contrast, no peaks with retention times similar to the toxin standards were observed for 18-1NT for GTX and C toxins (Fig. 4). A peak with a retention time fairly close to that of STX was detected for this strain (noted as peak X in Fig. 5). However, peak X was consistently offset from the standard, eluting slightly after it. The STX peak in the standard showed good reproducibility in its retention time and never coincided with peak X. To confirm that peak X

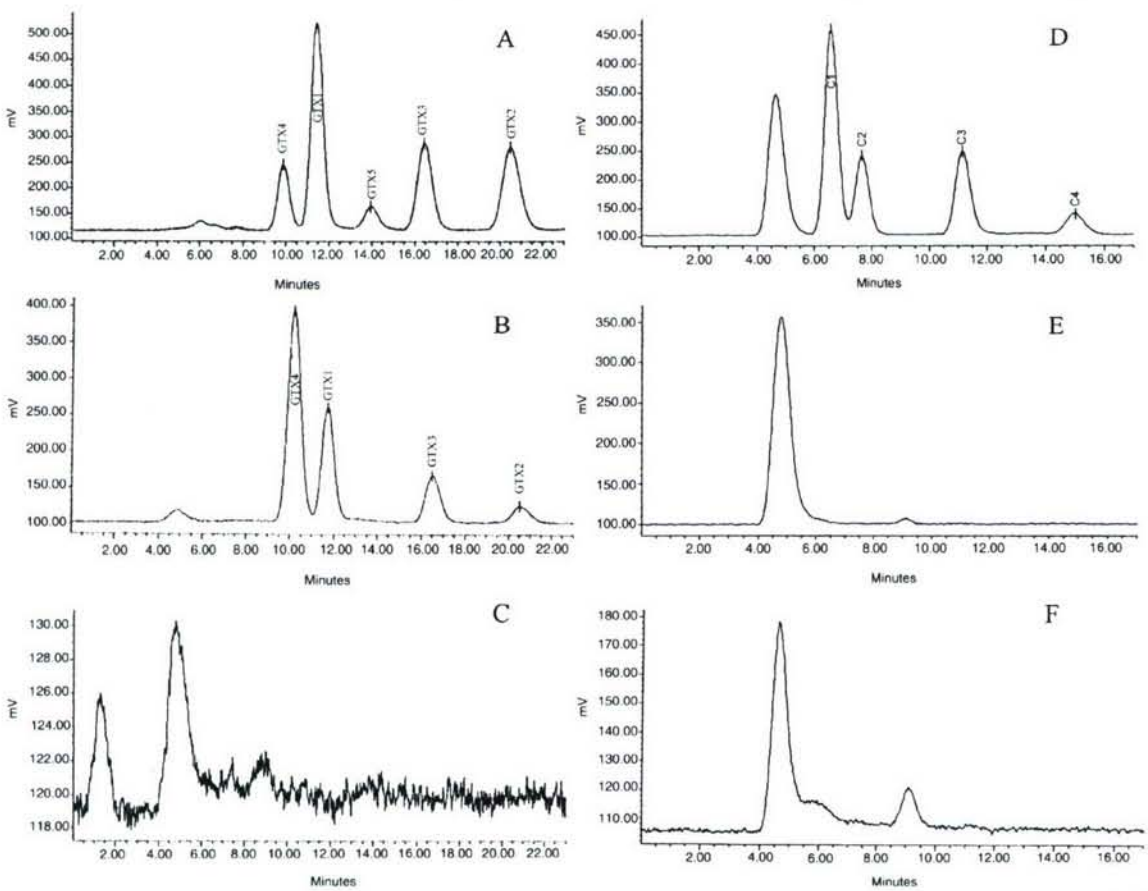

Fig. 4. HPLC analysis for GTX—(left) and C-toxins (right) in f/2 medium. Panels A and D refer to the standard mixture, B and E are 18-1T extracts and C and F refer to the 18-1NT culture.

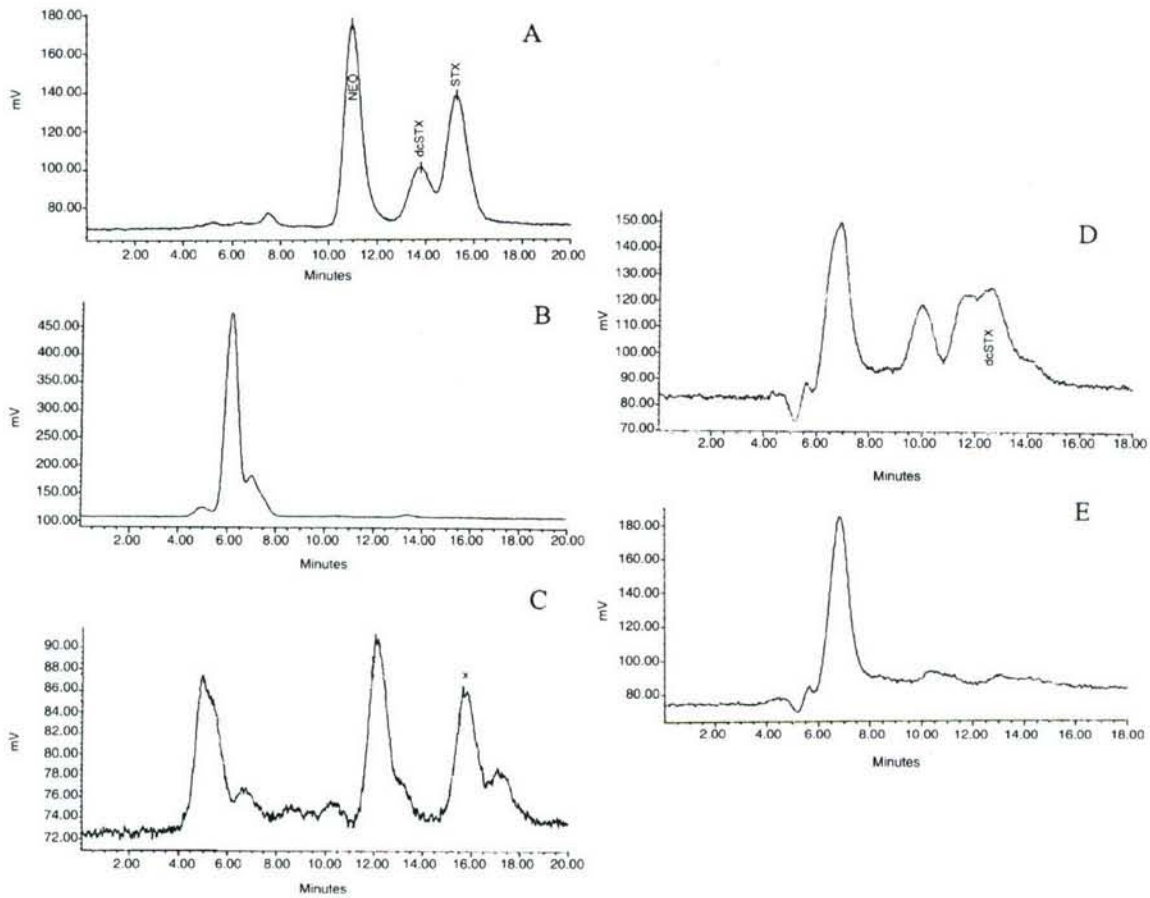

Fig. 5. HPLC analysis for neoSTX, dcSTX and STX for 18-1T and 18-1NT (B and C) in f/2 medium, and standard mixture (A). Putative STX peak in 18-1-NT extracts is labelled as 'x'. Results of the one hour hot hydrolysis in HCl (1:1 v/v) are shown in panel D (STX standard) and E (18-1NT extract).

was not STX, both sample and standard (containing only STX) were incubated at 100 °C in concentrated HCl (1:1 v/v) for 1 h. HPLC analysis of the concentrated, hotacid-treated samples showed different behaviors for the standard and 18-1NT sample (Fig. 5). For the hydrolyzed STX standard, the peak corresponding to STX disappeared and was converted to a compound with a retention time equivalent to that of decarbamoyl STX (dcSTX). The 18-1NT sample peak also disappeared, but did so without giving rise to dcSTX like compound. These differences in behavior following hydrolysis, plus the difference in retention times between peak X and STX lead to the conclusion that the former is in fact an 'impostor toxin' (Sato, 1998).

Analysis of 18-1T cultures grown in f/2 versus ASP7 media showed the former to be more toxic with 3.62 fmol cell^{-1} versus 1.22 fmol cell^{-1} in ASP7. The toxin profiles obtained (Fig. 6) were similar: both samples analyzed had GTX 1,4 as main toxins present, with lower quantities of GTX2,3 also detected. The mole percentages of each toxin differed slightly, as GTX2,3 accounted for 4% of the toxin content of the culture in ASP7 versus 7% in the culture grown in f/2. For 18-1NT, no peaks were ascribed to PSP toxins in ASP7 media.

Mouse neuroblastoma assay. In this assay, a comparison of sodium channel blocking (SCB) activity between the cell extracts of both cultures and a known non-toxic dinoflagellate, *Scrippsiella* sp. was performed. The MNA was used as a qualitative assay due to its high sensitivity for toxin detection by comparing the samples with the control. SCB activity, significantly higher than that of the controls, was detected for *A. lusitanicum*18-1T once cytotoxic effects were diluted out. The SCB activity detected could not be explained solely as matrix effects, as it differed significantly from that of the control extracts (Fig. 7, left) and the parental strain (Fig. 7, right). The residual levels of SCB activity seen in the controls are common in non-toxic extracts and media and correspond to the effect of co-extracted salts and other cellular components that have an

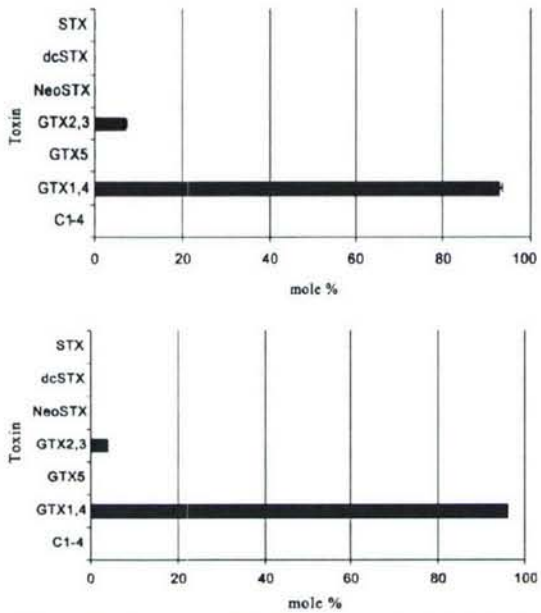

Fig. 6. Comparison of the toxin profile of the 18-1T *A. lusitanicum* cultures grown in different media in mol%. Top: toxin profile in mol% of 18-1T culture grown in f/2 medium. Bottom: toxin profile of the same culture in ASP7 medium.

effect on mouse neuroblastoma cells (Gallacher and Birkbeck, 1992).

Lower dilutions of the 18-1T sample showed a different behavior when compared to the negative control and the higher dilutions of this sample (Jellet et al., 1992). These effects can be ascribed to cytotoxic effects that could be diluted out of the sample. The cell pellets obtained from 18-1T subcultures growing in f/2 and ASP7 media showed similar results, as SCB activity was positively detected in both cases. However, the 18-1T culture grown in ASP7 media demonstrated a lower SCB activity, around 1/8th the value observed for cells growing in f/2.

No SCB activity ascribed to neurotoxins was detected for the 18-1NT culture, as the activity detected was always consistently lower than or equal to that of the control (Fig. 7, right). This result holds true for both growth media tested.

4. Discussion

We have demonstrated, for the first time, that saxitoxin production is not a constitutive, stable characteristic of *Alexandrium*, but can be lost during routine culture maintenance over a prolonged interval. Results clearly show a divergence, both in growth and toxicity, in two cultures derived from the same parental *A. lusitanicum* culture, originally isolated in 1962. Despite the fact both 18-1NT and 18-1T were grown and tested under the same conditions, no toxicity could be detected in the former, while the latter was always toxic, conserving the same toxin composition and roughly the same toxin content as the original 18-1 parental isolate, as analyzed in the past by Cembella et al. (1987), Alvito et al. (1995) and Mascarenhas et al. (1995). This striking loss of toxicity was confirmed by three independent toxin detection methods: the standard AOAC mouse bioassay, the mouse neuroblastoma assay and HPLC. Considering that the MNA has a higher level of sensitivity than HPLC (Gallacher and Birkbeck, 1992) and that the extracts assayed were from concentrations of cells well above the sensitivity limits described in the literature (Oshima, 1995), it is unlikely that the 18-1NT strain has low, undetected levels of toxicity, but this is of course possible. A number of independent sequence analyses of different rRNA domains also demonstrated that the toxic and non-toxic cultures are genetically identical for those markers examined. This strongly argues that the loss of toxicity is not due to a culturing artifact or mistake, such as culture mislabeling or accidental contamination with a different, non-toxic, species. These results and their implications are discussed in more detail below.

The longstanding view that saxitoxin production is a stable constitutive characteristic of an *Alexandrium* strain is refuted by the results obtained for our 18-1NT culture. Until our present study, only changes in the toxin content and more rarely in the toxin profile had been reported in the literature,

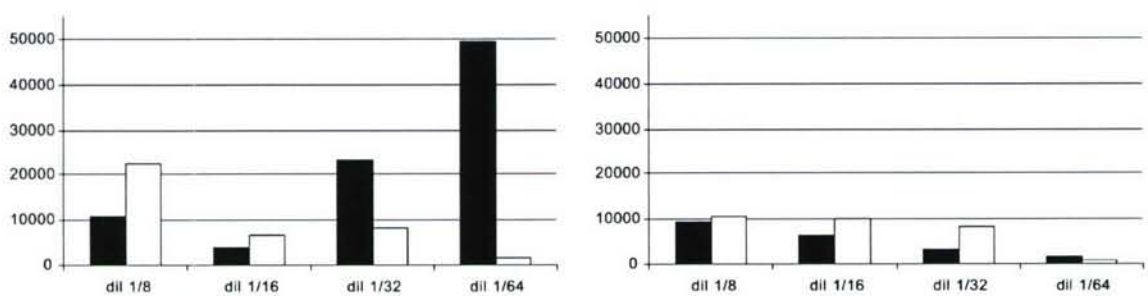

Fig. 7. Sodium channel blocking activity of dinoflagellate extracts (in black) and non-toxic control, *Scripsiella* (in white) obtained by mouse neuroblastoma assay. All cultures were grown in f/2 medium. Left: 18-1T; Right: 18-1NT.

while the ability to produce PSP toxins was never fully lost and it was never possible to elicit toxin production in non-toxic strains. This is therefore the first report of a species of *Alexandrium* losing the ability to produce toxins and it is also the first time any saxitoxin producer from any genus has ever become non-toxic. Three independent methods for toxin detection were used to confirm the loss of toxicity, each with different sensitivity limits and different principles of detection. Although the mouse bioassay has a detection limit of only 0.5 μM (Thibault et al., 1991), the HPLC system used has a sensitivity threshold of 14, 8, 3, and 14 nM for GTX1 to 4 in a 10 μl injection, respectively (D. Kulis, unpub. data). The qualitative assay (MNB) is approximately two orders of magnitude more sensitive than the mouse bioassay (Gallacher and Birkbeck, 1995). Within these limits, we are confident 18-1NT does not produce PSP toxins. The different toxin extraction methods, detection limits and toxin standards used on these methods explain the general lack of numerical agreement in the amount of toxin analyzed, though all three methods were in agreement that 18-1T was toxic and 18-1NT non-toxic. Samples for toxin analysis were always taken in exponential phase, when toxin content is highest in *Alexandrium* cultures (Anderson et al., 1990b; Franco et al., 1995; Mascarenhas et al., 1995), and large numbers of cells were extracted to ensure that instrument sensitivity or limits of detection were not issues. One putative STX peak was detected in extracts of 18-1NT using HPLC, but this peak did not behave like STX upon acid hydrolysis, nor was any SCB activity detected in extracts from this culture using the highly sensitive MNA. Similarly, the mouse bioassay did not detect any toxicity in 18-1NT. Furthermore, in our analysis of 18-1T and in all previous HPLC studies of the 18-1 strain of *A. lusitanicum*, STX has never been detected. We are therefore confident that peak X (Fig. 5) is an impostor toxin (Sato and Shimizu, 1998) and that 18-1NT is non-toxic. The biochemical nature of compound X is not known; it was present in only the non-toxic cultures, and has not been seen in HPLC analysis of other non-toxic *A. minutum* analyzed with the same methods and instrument (unpub. data).

The proof that the ability to produce toxin was completely lost was essential to our study. The partial reduction of toxin content in long-term cultures has been previously reported (Hansen et al., 2003) and still constitutes an argument for constitutive toxin production. Our present results, however, clearly show that it is possible for *Alexandrium* to lose the ability to produce saxitoxins.

The toxin profile of the 18-1T subculture was comparable to that previously obtained for the original *A. lusitanicum* 18-1 isolate (Mascarenhas et al., 1995; Cembella et al., 1987; Alvito et al., 1995). In all cases, GTX1,4 are dominant, with lower proportions of GTX2,3 also present. This toxin profile has therefore remained constant after 40 years in culture in 18-1T, but was dramatically altered in 18-1NT.

The loss of toxicity in the 18-1NT strain seems to be associated with a reduction in growth capability. The maximum cell yield, as well as the growth rate, were lower for this non-toxic strain compared to the toxic 18-1T strain in both media types tested. The growth rate and cell yield of cells of both strains kept in ASP7 were consistently lower than for the same cultures grown in f/2.

Toxin content and to a lesser extent, toxin profile also differed for 18-1T cells kept in the two media types. 18-1T culture had lower levels of toxicity when grown in the artificial ASP7 medium, achieving toxin content values that were lower than ever reported for this strain (Cembella et al., 1987; Mascarenhas et al., 1995). Low toxicity levels for *A. lusitanicum* subcultures kept in different growth media have been reported previously (Reguera et al., 1993). It is unclear what limits dinoflagellate growth and toxin production in ASP7. This medium differs from f/2 in some characteristics, namely nitrate and phosphate concentrations but the most significant difference probably relates to the artificial seawater base of ASP7 and either the lack of some unknown micronutrient or the presence of toxic contaminants from the reagent salts. These results demonstrate that toxin production could not be restored by growing the 18-1NT clone in optimal growth media (f/2), nor did 18-1T become non-toxic in the artificial media. This evidence strongly supports our view that the different media the clones were kept in was not responsible for the observed change. Growth of the 18-1NT clone could be improved by transferring it into f/2 media, but it never regained the ability to produce saxitoxins.

The observed differences in growth and toxicity might suggest that the two subcultures are not derived from the same parental strain, as might happen due to contamination or mislabeling of the parent culture during laboratory handling and culture transfers, for example. Throughout the interval in question, however, only a single *Alexandrium* strain (18-1) was cultured in the Laboratorio de Microbiologia e Ecotoxicologia where the loss of toxicity was first observed (Pereira et al., 2000). In other words, there was no morphologically similar culture to be mislabeled or to serve as a contaminant. Furthermore, our morphological and sequencing data both argue that 18-1NT and 18-1T have identical origins. Detailed thecal plate examinations showed that both cultures are morphologically identical, and that both have the distinctive taxonomic characteristics of *A. lusitanicum*. Sequencing of the D1/D2 region of rDNA also showed the two clones to be indistinguishable over 665 bp. In comparison, a detailed examination of rDNA data for *A. minutum* and *A. lusitanicum* (Scholin et al., 1994) showed these two morphologically similar species to differ by a single base pair over this same region. This single base difference between *A. lusitanicum* and *A. minutum* clones was also observed in this study. Using the same analysis, our

A. lusitanicum 18-1NT and 18-1T subcultures' D1/D2 sequences were identical.

The nucleotide sequences of the D8/D10 hypervariable domain of the Lsu rDNA were identical for the two clones, as was also found when the highly variable ITS1, 5.8S and ITS2 regions of rDNA were sequenced. Furthermore, when the ITS sequences of the 18-1NT and 18-1T strains were matched to the sequence for *A. lusitanicum* described by Penna and Magnani (1999) and posted in GenBank, a 100% match was found. All of these sequencing efforts argue that the 18-1NT and 18-1T are indeed from the same parental stock culture, and are *A. lusitanicum*. Nuclear ribosomal DNA is an ideal phylogenetic marker, as it possesses the same function in all organisms and has highly conserved regions (Adachi et al., 1996). Due to these characteristics, if 18-1T and NT are derived from the same culture and kept apart for only 10 years, base pair differences are not expected for these rDNA regions.

Other, more variable, regions of the genome, however, could have suffered and accumulated mutations over this time period. In view of the present results, we are left to speculate how the changes in toxicity and growth may have arisen. We find it noteworthy that the loss of toxicity is also associated with reduced growth potential. If indeed loss of toxicity is due to a mutation in a gene (or genes) involved in the toxin biosynthetic pathway, then the mutation responsible for this change (if such is indeed the case), is also affecting some pathway or pathways critical to cell growth and/or division. The fact that the parental culture has remained non-toxic for at least 4 years presents an argument for mutation rather than differential regulation at the gene expression level and that the loss of toxicity is not a transient characteristic.

Another possibility relates to the idea first proposed by Silva (1962) that bacteria are involved in saxitoxin production, either by autonomous toxigenesis or by supplying necessary metabolites. This concept has been advanced in several studies (see Gallacher and Smith, 1999 for a review). The occurrence of intracellular bacteria underneath the theca of the original 18-1 culture has been observed by electron microscopy (Franca et al., 1995) and bacteria have been isolated from aliquots of both 18-1NT and 18-1T cultures (data not shown). It is therefore possible that the continued antibiotic treatment of only the 18-1 culture eliminated associated bacteria essential for toxin production. Previous reports suggest the importance of associated bacteria in dinoflagellate (Doucette and Powell, 1998) and even diatom toxicity (Bates et al., 1995). In the case of dinoflagellates, however, authors report the production of substantially less toxin, rather than the total loss of toxicity in axenic cultures. The total loss of dinoflagellate toxicity due to removal of associated bacteria has never been reported and is therefore unlikely, but still possible, to provide an adequate explanation for our present results.

On the other hand, the sustained use of antibiotics could have influenced the cells in other ways. Despite affecting mainly protein synthesis and the prokaryotic cell wall, some of the antibiotics contained in the AM9 mixture with which the 18-1 culture was treated have been shown to be detrimental to algal cells. In particular, streptomycin caused sustained loss of pigmentation in *Chlorella* (Dube, 1952) and changes in chloroplast morphology and number, and a reduction in growth in *Euglena*, even after multiple transfers in streptomycin-free culture medium (Provasoli et al., 1948). Chloramphenicol, another antibiotic in the AM9 mixture, is reported to inhibit the growth of *Euglena* even at low concentrations (Miyoshi and Tsubo, 1969). It also can affect amino acid incorporation in the chloroplast system and inhibit chlorophyll formation. Interestingly, in that study, cells bleached by the chloramphenicol treatment never regained their green pigmentation after more than 2 years of transfers in medium without the antibiotic.

The effect of antibiotic treatments on dinoflagellate cells has not been documented, so it is possible, but still speculative, that prolonged exposure to the AM9 antibiotic mixture could have caused changes in the parental 18-1 culture, rendering it non-toxic. At that time, the 18-1T subculture was being maintained at another culture facility, but without the monthly antibiotic treatments (G. Doucette, pers. comm.), and thus it would not have been exposed to a similar stressor. The reduction in growth of *Euglena* observed by (Miyoshi and Tsubo, 1969) and (Provasoli et al., 1948) agrees with our own observation of reduced growth in 18-1NT, which was exposed to antibiotics for a longer time period.

The mechanisms underlying the reported changes in toxin production and growth are presently under investigation for the 18-1NT and 18-1T cultures.

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References

Adachi, M., Sako, Y., Ishida, Y., 1996. Analysis of *Alexandrium* (Dinophyceae) species using sequences of the 5.8S ribosomal DNA and internal transcribed spacer regions. J. Phycol. 32, 424–432.

Alvito, P., Sousa, I., Franca, S., Mascarenhas, V., 1995. Estudo experimental da relacao entre salinidade e toxicidade em

culturas de *Alexandrium lusitanicum*. IV Reunion Iberica sobre Fitoplancton Toxico y Biotoxinas, pp. 77–82.

Anderson, D.M., 1990. Toxin variability in Alexandrium species. In: Granéli, E., Sundström, B., Edler, L., Anderson, D.M. (Eds.), Toxic Marine Phytoplankton, Elsevier, New York, pp. 41–51.

Anderson, D., Kulis, D., Sullivan, J., Hall, S., Lee, C., 1990a. Dynamics and physiology of saxitoxin production by the dinoflagellates *Alexandrium* spp. Mar. Biol. 104, 511–524.

Anderson, D.M., Kulis, D.M., Sullivan, J.J., Hall, S., 1990b. Toxin composition variations in one isolate of the dinoflagellate *Alexandrium fundyense*. Toxicon 28 (8), 885–893.

Anderson, D.M., Kulis, D.M., Sullivan, J.J., Hall, S., Lee, C., 1990c. Dynamics and physiology of saxitoxin production by the dinoflagellates *Alexandrium* spp. Mar. Biol. 104 (3), 511–524.

Anderson, D.M., Kulis, D.M., Doucette, G.J., Gallagher, J.C., Balech, E., 1994. Biogeography of toxic dinoflagellates in the genus *Alexandrium* from the northeastern United States and Canada. Mar. Biol. 120 (3), 467–478.

AOAC, 1980. Standard mouse bioassay for paralytic shellfish toxins. In: Horwitz, W., (Ed.), Offical Methods of Analysis, Association of Official Analytical Chemists, Washington DC, pp. 298–299.

Balech, E., 1995. The genus *Alexandrium Halim* (Dinoflagellata). Sherkin Island Marine Station, Sherkin Island, Co. Cork, Ireland.

Bates, S.S., 1998. Ecophysiology and metabolism of ASP toxin production. In: Anderson, D.M., Cembella, A.D., Hallegraeff, G.M. (Eds.), Physiological Ecology of Harmful Algal Blooms, Springer, Heidelberg, pp. 405–426.

Bates, S.S., Douglas, D.J., Doucette, G.J., Léger, C., 1995. Enhancement of domoic acid production by reintroducing bacteria to axenic cultures of the diatom *Pseudo-nitzschia multiseries*. Nat. Toxins 3 (6), 428–435.

Burkholder, J.M., Glasgow, H.B., 1997. *Pfiesteria piscicida* and other *Pfiesteria*-like dinoflagellates: behavior, impacts, and environmental controls. Limnology and Oceanography 42, 1052–1075.

Cembella, A.D., 1998. Ecophysiology and metabolism of paralytic shellfish toxins in marine microalgae. In: Anderson, D.M., Cembella, A.D., Hallegraeff, G.M. (Eds.), Physiological Ecology of Harmful Algal Blooms, G 41, Springer, Heidelberg, pp. 381–403.

Cembella, A.D., Sullivan, J.J., Boyer, G.L., Taylor, F.J.R., Andersen, R.J., 1987. Variation in paralytic shellfish toxin composition within the *Protogonyaulax tamarensis/catenella* species complex; red tide dinoflagellates. Biochem. Syst. Ecol. 15 (2), 171–186.

Chinain, M., Germain, M., Sako, Y., Pauillac, S., Legrand, A., 1998. Genetic diversity in French Polynesian strains of the ciguatera-causing dinoflagellate *Gambierdiscus toxicus*: RFLP and sequence analysis on the SSU and LSU rRNA genes. In: Reguera, B., Blanco, B., Fernandez, M.L., Wyatt, T. (Eds.), Harmful Algae, Xunta de Galicia and IOC, Paris, France, pp. 287–290.

D'Onofrio, G., Marino, D., Blanco, L., Busico, E., Montresor, M., 1999. Toward an assessment on the taxonomy of dinoflagellates that produce calcerous cysts (Calciodinelloideae, Dinophyceae): a morphological and molecular approach. J. Phycol. 35, 1063–1078.

Doucette, G.J., Powell, C., 1998. Algal–bacteria interactions: can they determine the PSP-related toxicity of dinoflagellates. In: Reguera, B., Blanco, J., Fernandez, M.L., Wyatt, T. (Eds.), Harmful Algae, Xunta de Galicia and IOC, pp. 406–409.

Doyle, J.J., Doyle, J.L., 1990. Isolation of plant DNA from fresh tissue. Focus 12, 13–15.

Dube, J.F., 1952. Observation on chlorophyll deficient strain of *Chlorella vulgaris* obtained after treatment with streptomycin. Science 116, 278.

Flynn, K.J., Flynn, K., John, E.H., Reguera, B., Reyero, M.I., Franco, J.M., 1996. Changes in toxins, intracellular and dissolved free amino acids of the toxic dinoflagellate *Gymnodinium catenatum* in response to changes in inorganic nutrients and salinity. Journal of Plankton Research 18 (11), 2093–2111.

Franca, S., Viegas, S., Mascarenchas, V., Pinto, L., Doucette, G.J., Lassus, P., Arzul, G., 1995. Prokaryotes in association with a toxic *Alexandrium lusitanicum* in culture. In: Gentien, P., Marcaillou-Le Baut, C. (Eds.), Harmful Marine Algal Blooms/Proliferation d'Algues Marines Nuisibles, Proceedings of the Sixth International Conference on Toxic Marine Phytoplankton, Nantes (France), October 1993, Lavoisier, Paris (France), pp. 45–51.

Franco, J.M., Fraga, S., Zapata, M., Bravo, I., Fernandez, P., Ramilo, I., 1995. Comparison between different strains of genus *Alexandrium* of the minutum group. In: Lassus, P., Arzul, G., Erard-Le Denn, E. (Eds.), Harmful Marine Algal Blooms/Proliferation d'Algues Marines Nuisibles, Proceedings of the Sixth International Conference on Toxic Marine Phytoplankton, Nantes (France), October 1993, Lavoisier, Paris (France), pp. 53–58.

Fritz, L., Triemer, R.E., 1985. A rapid simple technique utilizing Calcofluor white M2R for the visualization of dinoflagellate thecal plates. J. Phycol. 21 (4), 662–664.

Gallacher, S., Birkbeck, T.H., 1992. A tissue culture assay for the direct detection of sodium channel blocking toxins in bacterial culture supernates. FEMS Microbiol. Lett. 92, 101–108.

Gallacher, S., Smith, E., 1999. Bacteria and paralytic shellfish toxins. Protist 150, 245–255.

Guillard, R.R.L., Ryther, J.H., 1962. Studies of marine plankton diatoms I. *Cyclotella nana* Hustedt and *Detonula confervacea* (Cleve) Gran. Can. J. Microbiol. 8, 229–239.

Hall, S., 1982. Toxins and toxicity of *Protogonyaulax* from the Northeast Pacific. University of Alaska, Fairbanks.

Hansen, G., Daugbjerg, N., Franco, J.M., 2003. Morphology, toxin composition and LSU rDNA phylogeny of *Alexandrium minutum* (Dinophyceae) from Denmark, with some morphological observations on other European strains. Harmful Algae 2 (4), 317–335.

Jellet, J.F., Marks, L.J., Stewart, J.E., Dorey, M.L., Watson-Wright, W., Lawrence, J.F., 1992. Paralytic shellfish poison (saxitoxin family) bioassays: automated endpoint determination and standardization of the in vitro tissue culture bioassay and comparison with the standard mouse bioassay. Toxicon 30, 1143–1156.

Mascarenhas, V., Alvito, P., Franca, S., Sousa, I., Rodriguez-Vasquez, J.A., Martinez, A.G., 1993. The dinoflagellate *Alexandrium lusitanicum* isolated from the coast of Portugal: observations on toxicity and ultrastructure during growth phases. In: Lassus, P., Arzul, G., Erard-Le Denn, E., Gentien, P., Marcaillou-Le Baut, C. (Eds.), Harmful Marine Algal Blooms/Proliferation d'Algues Marines Nuisibles, Proceedings of the Sixth International Conference on Toxic Marine

Phytoplankton, Nantes (France), October 1993, Lavoisier, Paris (France), pp. 71–76.

Miyoshi, Y., Tsubo, Y., 1969. Permanent bleaching of *Euglena* by chloramphenicol. Plant Cell Physiol. 10, 221–225.

Oshima, Y., 1995. Post-column derivatization HPLC methods for paralytic shellfish poisons. In: Hallegraeff, G.M., Anderson, D.M., Cembella, A.D. (Eds.), Manual on Harmful Marine Mcroalgae. IOC Manuals and Guides, No. 33, UNESCO, Paris, pp. 81–94.

Penna, A., Magnani, M., 1999. Identification of *Alexandrium* (Dinophyceae) species using PCR and rDNA-targeted probes. J. Phycol. 35, 615–621.

Pereira, P., Andrinolo, D., Sam-Bento, F., Alvito, P., Martins, C., Franca, S., 2000. Novos resultados sobre estudos de toxicidade PSP em dinoflagelados e bacterias associadas. VI Reunion Iberica sobre Fitoplancton Toxico y Biotoxinas, pp. 139–148.

Provasoli, L., Hutner, S., Schatz, A., 1948. Streptomycin-induced chlorophyll-less races of *Euglena*. Proc. Soc. Exp. Biol. Med. 69, 279–282.

Provasoli, L., 1963. Growing marine seaweeds. Fourth International Seaweed Symposium, pp. 9–17.

Reguera, B., Franco, J., Fernandez, P., 1993. Datos preliminares sobre el crecimiento y la produccion de toxinas PSP en cultivos de dos cepas de *Alexandrium lusitanicum*. III Reunion Iberica sobre Fitoplancton Toxico y Biotoxinas, pp. 97–99.

Sato, S., Shimizu, Y., 1998. Purification of a fluorescent product from the bacterium *Moraxella*: a neosaxitoxin impostor. In: Reguera, B., Blanco, J., Fernandez, M.L., Wyatt, T. (Eds.), Harmful Algae, Xunta de Galicia and IOC, pp. 465–467.

Scholin, C., Anderson, D., 1994. Identification of group-/and strain-specific generic markers for globally distributed *Alexandrium* (Dinophyceae). I. RFLP analysis of SSU rRNA genes. J. Phychol. 30, 744–754.

Scholin, C.A., Herzog, M., Sogin, M., Anderson, D.M., 1994. Identification of group-and strain-specific genetic markers for globally distributed *Alexandrium* (Dinophyceae). 2. Sequence analysis of a fragment of the LSU rRNA gene. J. Phycol. 30 (6), 999–1011.

Silva, E.S., 1962. Some observations on marine dinoflagellate cultures. I. *Prorocentrum micans* Ehr., *Gymnodinium spinifera* (Clap. and Lach.) dies., *Gonyaulax tamarensis* Leb., and *Peridinium trochoideum* (Stein) Lemm. Notas e estudos do Inst Biol. Mar. 26, 1–21.

Silva, E.S., Sousa, I., 1981. Experimental work on the dinoflagellate toxin production. Separata dos Arquivos do INSA VI, 381–387.

Sousa Silva, E., 1990. Intracellular bacteria: the origin of dinoflagellate toxicity. J. Environ. Pathol. Toxicol. Oncol. 10 (3), 124–128.

Springer, J., Interactions between two commercially important species of bivalve molluscs and the toxic dinoflagellate, *Pfiesteria piscicida*, North Carolina State University, 2000.

Stoecker, D., Parrow, M., Burkholder, J., Glasgow, H. Jr., 2002. Grazing by microzooplankton on *Pfiesteria piscicida* cultures with different histories of toxicity. Aquat. Microb. Ecol. 28, 79–85.

Thibault, P., Pleasance, S., Laycock, M.V., 1991. Analysis of paralytic shellfish poisons by capillary electrophoresis. J. Chromatogr. 542, 483–501.

Utermöhl, H., 1958. Neue Wege in der quantitativen Erfassung des Planktons (mit besonderer Berücksichtigung des Ultra-planktons. Verh. int. Ver. theor. angew. Limnol. 5, 567–596.

CHAPTER 3

ROLE OF BACTERIA ASSOCIATED WITH TOXIC AND NON-TOXIC SUBCULTURES OF AN *ALEXANDRIUM LUSITANICUM* STRAIN

ABSTRACT

Saxitoxins, the causative agent of the human syndrome paralytic shellfish poisoning, are produced, among others, by several species of dinoflagellates of the genus *Alexandrium*. Previous work has demonstrated the existence of two *Alexandrium lusitanicum* subcultures from the same parental strain that are clonal but that differ in the ability to produce saxitoxins. In addition to toxin production, growth is also negatively affected in the non-toxic sub-culture compared to the toxic one. In view of the sustained use of antibiotics in the non-toxic clone's growth medium, changes in bacterial community were hypothesized as being implicated in the loss of toxicity and altered growth.

Denaturing gradient gel electrophoresis (DGGE) patterns confirmed the differences in bacterial assemblages of the two subcultures, each of which has a complex bacterial assemblage. Ribosomal clone library sequencing showed that the majority of the clones identified for the two dinoflagellate cultures were associated with the phylum Bacteroidetes, with remaining clones belonging to the Alphaproteobacteria. However, while the totality of the Alphaproteobacteria in the toxic clone are associated with the Rhodobacteraceae, the majority of the clones identified in non-toxic culture belong instead to the Sphingobacteraceae family.

Bacteria were found to influence growth and toxin production to a certain extent, as removing bacteria from toxic subcultures reduced toxin production. Bacteria also were shown to influence growth, as axenic cultures consistently displayed an increased duration of the lag growth phase, though exponential growth rates were similar. However, adding bacteria from the toxic, faster growing culture alone or in combination with the assemblage from the non-toxic isolate, did not elicit toxin production, nor did it affect the reduced growth observed for the non-toxic isolate. In conclusion, associated bacteria do not seem to be the main factor underlying the difference in toxicity of the two subcultures.

INTRODUCTION

Paralytic shellfish poisoning (PSP) is the most widespread of the human poisoning syndromes linked to harmful algal blooms (HAB). Saxitoxins, the causative agent of PSP, are produced by several genera of dinoflagellates and are transferred to higher trophic levels through accumulation by filter feeding bivalves and fish. Saxitoxins have also been identified in several species of freshwater cyanobacteria (Humpage et al. 1994), Charmicheal et al 1997, Pereira et al, 2000)

The genus *Alexandrium* contains a number of dinoflagellate species that have been conclusively linked to PSP episodes in various regions of the world (Cembella 1998). However, while the causative agents of PSP are known, the mechanisms implicated in toxin production and the manner in which physical and biological factors affect the severity and nature of PSP episodes are poorly understood. Studies addressing the mechanisms of toxin production have been limited to analyzing related *Alexandrium* strains that do and do not exhibit toxin production. This approach has been largely unsuccessful in identifying key genes or pathways, presumably due to other differences between the toxic strains and the closely related non-toxic strains to which they are compared.

Previous work (Martins et al. 2004) demonstrated the existence of two *Alexandrium lusitanicum* subcultures of the same parent strain that are clonal but that differ in terms of their ability to produce saxitoxins. The lack of toxicity was confirmed by three independent methods (mouse bioassay, high performance liquid chromatography (HPLC) and mouse neuroblastoma assay). Not only toxin production but also growth was negatively affected in the non-toxic culture (clone 18-1NT) compared to the toxic clone (18-1T). 18-1NT consistently had a slower growth rate and attained a lower maximum cell density under all tested growth conditions. This pair of cultures is an ideal experimental system in which to study the mechanisms involved in growth and toxin production, as only growth and the ability to produce saxitoxins are thought to vary between the two subcultures.

The culturing history of the two subcultures is well documented and can potentially shed light on the mechanisms causing the changes in 18-1NT toxicity and growth. The original culture, designated 18-1, was isolated in 1962 from Obidos Lagoon, Portugal, and kept in Provasoli's ASP7 artificial seawater medium (Provasoli 1963). As standard procedure, the culture was transferred during exponential phase to new media and treated with AM9 antibiotics (Silva and Sousa 1981) every two transfers. In 1992, a subculture of this isolate was established and sent to Dr. G. Doucette's Laboratory in Charleston, SC, USA. This subculture was no longer treated with antibiotics and was switched to f/2 growth medium (Guillard and Ryther 1962). In 2000, routine analyses showed the parental isolate (re-named 18-1NT) had lost the ability to produce saxitoxins, while the subculture (18-1T) retained roughly the same toxin content and toxin profile.

In view of this information, several hypotheses were considered for the loss of toxin production and the different growth capabilities of the 18-1 clones. The main difference between the maintenance conditions of the two subcultures involved the regular use of antibiotics in the non-toxic clone's growth medium. The mixture contained a combination of several antibiotics which would have affected specific bacteria, potentially altering the bacterial assemblage of 18-1NT. The change of bacterial community was thus suspected as a factor influencing the changes in growth and toxin production observed in the experimental system.

Silva (1962) first proposed a role of bacteria in dinoflagellate toxin production, and this concept has been explored in subsequent studies (see (Gallacher and Smith 1999) for a

review). While a direct bacterial contribution to saxitoxin production has been discounted in most cases (Baker et al. 2003; Martins et al. 2003), there is still evidence that prokaryotes somehow influence growth and saxitoxin production in dinoflagellate cultures. However, there is little agreement on the level of the effect, as results vary significantly between studies and between different species of dinoflagellates. Furthermore, a single bacteria species has been shown to affect toxin production, as has a mix of species either isolated from the alga itself or from other species (Doucette 1995).

Despite the uncertainties surrounding bacterial involvement in toxin production, the fact that bacteria co-exist with dinoflagellate cells is established beyond doubt, both in culture and in the field. The presence of bacteria living in close association with dinoflagellate cells, both extra and intra-cellularly, has been demonstrated for many species (Franca et al. 1995), (Alverca et al. 2002). Bacteria associated with dinoflagellates seem to be restricted to the Cytophaga-Flavobacterium-Bacteroides (CFB) and α- and γ-Proteobacteria groups of Eubacteria. Recent studies have added to the body of evidence that suggests a specific association between certain bacterial species and dinoflagellates (Jasti et al. 2005). Despite these observations, the specificity and the role of the relationship remains largely unknown.

To investigate the role of bacteria in the loss of toxicity and the slower growth of *Alexandrium lusitanicum* clone 18-1NT, the effect of differing bacterial assemblages were tested in subcultures of this species. This is a novel experimental system in which to study the effect of bacteria. If in fact bacteria are critical factors, we should expect to find different bacterial assemblages associated with the toxic and non-toxic cultures. Furthermore, toxin production might be re-established in the non-toxic clone by adding the bacterial assemblage associated with the toxic strain. To assess the effect of bacteria on 18-1NT, the bacteria of both subcultures were mixed. The experiments were designed to either replace the bacterial assemblage of the non-toxic subculture with that of the toxic one or to allow for the bacteria of the two subcultures to co-exist.

MATERIAL AND METHODS

Dinoflagellate cultures

Two cultures of the dinoflagellate *Alexandrium lusitanicum*, recently shown to be clonal isolates differing in toxicity and growth (Martins et al, 2004) were used in this study. Since 2000, both cultures have been maintained in the same growth conditions (f/2 growth medium, with no antibiotic addition). For all experiments, cultures were maintained in autoclaved borosilicate tubes containing 25 ml sterile growth medium at 20±1 °C on a 16:8 h L:D cycle with 200 $\mu mol.m^{-2}.s^{-1}$ irradiance.

Bacterial clone libraries

Cells from exponentially growing cultures of 18-1T and 18-1NT were collected by centrifugation (5000g, 10 minutes). The growth medium overlying the cell pellet was

completely discarded and the pellet washed once in sterile f/2 medium to avoid overrepresentation of bacteria existing in the medium. After centrifugation, cell pellets were flash frozen in liquid nitrogen and stored at -80° C prior to extraction.

DNA from the dinoflagellates and co-occurring bacteria was extracted from each cell pellet by a 3% CTAB method (Doyle and Doyle 1990) with slight modifications (Martins et al, 2004). Briefly, dinoflagellate cells were ruptured by bead beating with silica beads until the majority of dinoflagellate cells were confirmed to be lysed under a light microscope. An aliquot of the extracted DNA was checked in an agarose gel for integrity and quantified by comparison to a DNA mass ladder.

Prokaryotic universal primers UPf and UPr (Table 1) were used for PCR amplification of approximately 1500 base pairs of the 16S ribosomal DNA (rDNA). 50 µl PCR reactions containing approximately 25 ng of DNA were carried out in an ABI thermocycler. PCR conditions were as follows: 5 minute denaturing step at 96°C, followed by 25 cycles of 45 seconds denaturing at 95°C, 45 seconds annealing at 55°C, 1 minute extension at 72°C; and a final extension step of 7 minutes at 72°C. PCR reactions were purified using the MinElute PCR purification kit (Qiagen, Valencia, CA) and ligated into the PCR dual promoter-TOPO vector (Invitrogen, Carlsbad, CA). Cloning reactions were transformed into DH5α chemically competent cells (Invitrogen) following instructions from the manufacturer.

Colonies potentially containing sequence inserts were selected and plasmids were extracted using a Genemachines RevPrep Orbit. The size of each insert was estimated by digesting purified plasmids with the restriction enzyme EcoRI (New England BioLabs) at 37°C overnight. Products of the reaction were visualized in an agarose gel and only plasmids containing potential full length sequences were kept. These plasmids were sequenced using the T7 primer (for partial sequencing) or primers T7, Sp6, 907r, 787r and 518f (for full length sequencing) (Table 1). Sequencing reactions were completed with the Big Dye terminator and run in an ABI 3730XL capillary sequencer, following the protocol described in http://jbpc.mbl.edu/.

Sequences thus obtained were imported into the Sequencher software package (Gene Codes). Vector and primer sequences were removed and sequence quality was visually inspected. Resulting sequences were assembled into consensus of 98% similarity for further analysis. Consensus sequences were automatically aligned using the ARB (Ludwig et al. 2004) fast aligner tool followed by manual correction.

Chimeras formed during PCR amplification were removed from the alignment. Different approaches were used to ensure identification of these PCR artifacts. Firstly, sequences were analyzed through Chimera_check from RDP (Maidak et al. 1999) and Belerophon (Hugenholtz and Huber 2003). The final decision to exclude sequences was based on visual inspection of the alignment, as chimeras were assumed to originate exclusively from sequences truly present in the initial DNA sample (defined as sequences occurring

Table 3-1. Sequences of Bacteria specific primers used for sequencing, DGGE analysis and clone library construction.

Primer	Sequence (5' to 3')	Reference
UPf	AGA GTT TGA TYM TGG C	(Weisburg et al. 1991)
UPr	GYT ACC TTG TTA CGA CTT	(Weisburg et al 1991)
341F	CCT ACG GGA GGC AGC AG	(Schafer and Muyzer 2001)
907R	CCG TCA ATT CMT TTG AGT TT	(Schafer and Muyzer 2001)
518F	CCA GCA GCC GCG GTA AT	(Buchholz-Cleven et al. 1997)
Sp6	ATT TAG GTG ACA CTA TAG	(Invitrogen)
T7	TAA TAC GAC TCA CTA TAG GG	(Invitrogen)

more than twice). Sequences were also matched to the 16S rDNA secondary structure of *E. coli* to identify abnormalities and non-complementary helical regions.

Due to different lengths of sequences obtained in this study it was necessary to establish clusters of sequences for further phylogenetic analysis. A cluster was defined when sequences grouped in the same tree branch in multiple phylogenetic tree constructions. One sequence in each cluster was chosen for further full length sequencing and phylogenetic analysis.

Phylogenetic trees were constructed in PAUP (Swofford 2001). Closely related 16S rDNA sequences (as determined by BLASTn searches) and close type species were included in the initial alignment. Also included in the analysis were bacterial 16S rDNA sequences obtained from cultures of the dinoflagellates *Gymnodinium catenatum* (Green et al. 2004), *Alexandrium* and *Scripsiella* (Hold et al, 2001), *Prorocentrum lima* (Lafay et al. 1995) and *Noctiluca scintillans* (unpublished) stored in GenBank. Only sequences longer than 1100 base pairs were chosen to be included in the phylogenetic tree. Further phylogenetic classification of the different sequences was performed in RDP classifier tool in RDP. Neighbor joining trees with Jukes-Cantor correction are depicted in this study. Highly variable and end regions of the alignment were excluded from the phylogenetic analysis by designing filters in ARB. Other trees were constructed using the model of base pair substitutions that fits the data as chosen by Modeltest (Posada and Crandall 1998). For sequences belonging to the Alphaproteobacteria, a Tamura-Nei evolutionary model was chosen, while for the CFB sequences the general time reversible model was used.

Denaturing Gel Gradient Electrophoresis

Dinoflagellate cells and associated bacteria were collected and DNA extracted as above. A section of the 16S rDNA was amplified by PCR, using the universal primers 341F-GC and 907R (Table 1) and cycling conditions as described in (Schafer and Muyzer 2001). The amplified product was quantified by agarose gel electrophoresis and approximately 300 ng were loaded on a DGGE gel. At the onset of the study, it was observed that the DGGE banding pattern of dinoflagellate cultures consisted of dominant bands (defined as higher intensity under the UV light) and minor bands (bands of weaker intensity). The DNA concentration to load in each gel was chosen as to allow simultaneous visualization of minor bands as well as well-defined major bands.

DGGE was performed as described by Schafer and Muyzer (2001). A denaturing gradient ranging from 20 to 80% urea and formamide was chosen for all experiments. Gels were run at a constant temperature and voltage of 60°C and 100V, respectively. After 18 hours, gels were stained in SYBR Green (Molecular Probes, Invitrogen) for 1 hour and photographed under UV light.

Visible bands were excised from the gel and the DNA was extracted from the agarose by overnight elution in molecular grade water. Nucleic acids were re-amplified using 341F and 907R primers (Table 1) following conditions as described in Schafer and Muyzer

(2001). PCR products corresponding to individual bands were cloned and full length sequences obtained as described in the previous section.

Several aspects of the methodology were addressed so DGGE could be employed to adequately compare the microbial community of 18-1T and 18-1NT. To test the reproducibility of the method, each DNA extraction was amplified in three separate PCR reactions and loaded alongside in the DGGE gel to detect potential differences in the banding pattern due to PCR artifacts. Banding patterns and band intensity were highly reproducible in multiple PCR reactions. No differences were observed in the banding pattern of DNA extracts either kept at –20°C for several days prior to PCR amplification or when amplification was performed immediately following extraction.

Effect of associated bacteria

To determine the effect of bacteria on the dinoflagellate growth and toxicity: 1) bacteria associated with 18-1 sub-cultures were mixed, so that 18-1T will have its own bacterial assemblage plus the assemblage of 18-1NT, and vice-versa (referred to as mixing experiments); and 2) the bacterial assemblage of 18-1T was removed and substituted by the assemblage of 18-1NT, and vice-versa (referred to as cross-inoculation experiments).

1) Mixing experiments

A method of mixing was chosen that would ensure the bacteria assemblage of 18-1T and 18-1NT were as similar as possible and consisted of a mix of the two. The two 18-1 subcultures were allowed to grow in a mixing chamber, consisting of two glass flasks connected through a 5 μm filter, during a 50 day period. Each flask contained 100 ml of growth medium. Under these growth conditions, particles smaller than 5 μm could exchange freely between the two cultures, while dinoflagellate cells were excluded due to the filter. Aliquots of each culture were transferred into new f/2 medium 3 times during this period to avoid nutrient depletion and metabolite buildup. Samples were collected in late exponential phase of growth for toxin analysis, cell counts and DGGE analysis. Toxin content and DGGE profile of cultures with mixed bacterial assemblages were compared to the original 18-1T and 18-1NT cultures grown in isolation.

2) Cross-inoculation experiments

In order to perform the cross-inoculation experiments, the two clones had to first be rendered axenic. This was achieved by treating 1 ml of each 18-1 sub-culture with serial dilutions of antibiotic mixtures in 15 ml of f/2 media with NZY bacterial media, added to promote bacterial growth (J. Rooney-Varga, pers comm.). Cultures were incubated in the antibiotic mixture for 24 hours, after which a 1 ml aliquot was resuspended in sterile f/2 medium. Cultures were maintained in antibiotic free media for several culture transfers to dilute any remaining antibiotic mixture.

Individual cultures were routinely checked for bacterial contamination by PCR amplification using different sets of general prokaryotic primers (Table 1). DNA was

extracted from each culture in stationary phase following the conditions described previously. Different DNA concentrations were used as template in the PCR reactions to maximize the probability of detection of bacterial 16S rDNA. DNA of dinoflagellate cultures not treated with antibiotics was used as a positive control of DNA extraction and amplification.

Growth of cultures deemed axenic was recorded through *in vivo* fluorescence measurement (Turner Designs Model 10 fluorometer) at regular intervals. Samples were removed for DNA extraction, toxin analysis and cell counts at several stages of the growth curve, so that each growth stage is represented by several samples.

For cross-inoculation experiments, spent medium of 18-1T was added to axenic cultures of 18-1NT and vice-versa. Spent medium was defined as f/2 growth medium in which 18-1 cultures were maintained up to exponential phase, at which point dinoflagellate cells in culture were disrupted and removed by filtration. Cells were disrupted by briefly vortexing a 15ml aliquot of culture with glass beads (10 seconds, 3 times). This was done in order to release bacteria either attached to the dinoflagellate theca or those located intracellularly. The removal of whole dinoflagellate cells was insured by filtration through a 2.7 μm (Millipore) pore size filter which allowed for the passage of free bacteria. Growth and toxin content of cross-inoculated cultures was recorded at several stages of the growth curve, as described above. Growth rates and toxin content were compared to those of axenic cultures as well as the original non-axenic cultures maintained in the same growth conditions. In order to attribute potential differences in these parameters to the presence of bacteria and not metabolites carried over during the cross-inoculation of bacteria, spent media were filtered through a 0.2 μm filter and added to axenic cultures as controls.

Toxin analysis

Aliquots of a well-mixed culture were collected and preserved in Utermöhl's solution (Utermöhl 1958) for cell counts in a Sedgwick-Rafter counting chamber. Each count was made at least in duplicate and the average recorded.

Fifteen ml of each culture were harvested by centrifugation (5000g; 10 min) and cell pellets were ressuspended in 0.05M acetic acid. Toxins were extracted by sonicating (10W, three 10s cycles) the acid slurry in an ice bath followed by 3 cycles of freezing/ thawing. Samples were stored at -20°C prior to analysis.

Toxin content and profile of each sample was obtained by HPLC according to the method of Oshima (1995) as described in Anderson et al. (1994). PSP toxins were identified and quantified by comparison with reference standards, kindly provided by Y Oshima (Tohoku University, Japan). Toxicities of the derivatives (in fg STX equivalent·cell^{-1}) were calculated from the molar composition data using individual potencies in mouse units per μmol^{-1}. (Oshima 1995): C1 - 15; C2 - 239; C3 - 32; C4 - 143; GTX1 - 2468;

GTX2 - 892; GTX3 - 1584; GTX4 - 1803; GTX5 - 160; dcGTX2 - 382; dcGTX3 - 935; NEO - 2295; dcSTX - 1274; STX - 2483.

RESULTS

DGGE

DGGE was performed to determine if differences existed in the bacterial assemblages of 18-1T and 18-1NT. Banding patterns obtained reflect the genetic diversity of bacteria associated with both cultures (Fig.1). 18-1T and 18-1NT both have a complex bacterial assemblage as multiple DNA fragments could be separated by the denaturing gradient gel. No bands common to 18-1T and 18-1NT could be identified, confirming the differences in bacterial assemblages of the two 18-1 subcultures. The exception could be the band designated by * in Figure 1, though it is unclear if this represents the same sequence.

It was also recognized that temporal succession of the associated bacterial community could potentially confound the comparative analyses. The temporal variability of the associated bacteria of toxic and non-toxic 18-1 was assessed by sampling during lag, exponential and stationary growth stage of the dinoflagellate. The DGGE banding patterns of each 18-1 subculture in different growth stages were generally similar, with a few exceptions in bands of weaker intensity (Fig.2). As demonstrated in the case of 18-1T, there was 100% agreement for the banding patterns obtained for exponential and stationary phase. In comparison, in the lag phase one band was missing and there was also a band only visible in cultures in this phase. Samples taken from each culture at two different dates had the same pattern (Fig.2). Therefore the DGGE pattern discussed in this study was considered to be representative of the 18-1T and 18-1NT cultures, independent of growth stage.

All sequences recovered from the excised DGGE bands corresponded to bacterial 16S rDNA. However, because the DGGE primers were used for DNA amplification, only partial 16srDNA were available for further phylogenetic analysis. BLASTn search of the partial sequences revealed all to be affiliated either to the Alphaproteobacteria or the Bacteroidetes Phyla.

Bacterial clone library

Clone libraries were constructed to provide further information on the bacteria present in each 18-1 subculture. After chimeric sequences were eliminated, 166 partially sequenced clones were obtained from the 18-1T subculture and 126 from the 18-1NT culture. The majority of the clones were associated with the phylum Bacteroidetes with all other clones belonged to the Alphaproteobacteria class of the Proteobacteria (Fig.3). Only one clone sequenced from 18-1NT could not be classified by the RDP classifier in any phylum with a 95% level of confidence.

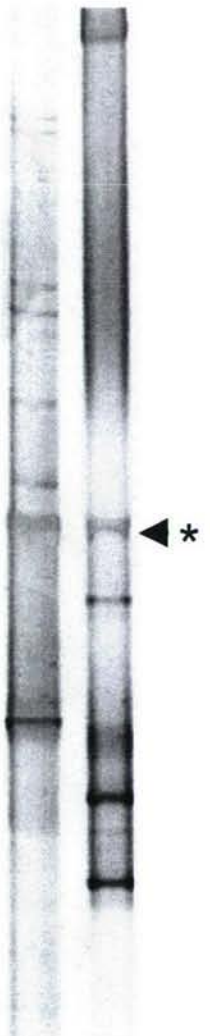

FIGURE 3-1. DGGE patterns of 16S rDNA gene fragments amplified from bacteria present in dinoflagellate cultures of 18-1T (left) and 18-1NT (right). Band indicated by the arrow could potentially be shared between both cultures.

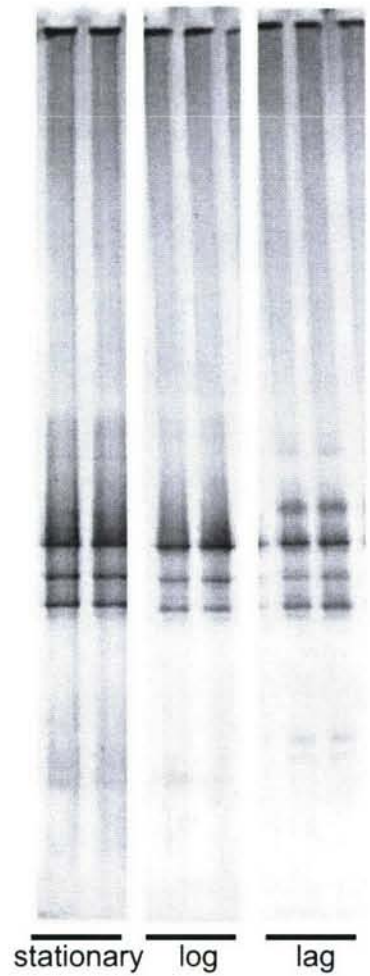

FIGURE 3-2. DGGE patterns of 16s rDNA fragments amplified from bacteria from 18-1T cultures during lag, log and stationary phases. The two profiles in each growth stage were obtained from two separate cultures of 18-1T, harvested 2 months apart.

Despite the similarities between the associated bacteria of toxic and non-toxic 18-1 at the phylum level, differences were observed in the composition of the Alphaproteobacterial component, determined through the RDP classifier tool (pre-determined level of confidence of 95%). While the totality of the Alphaproteobacteria in the toxic form of 18-1 are associated with the Rhodobacteraceae, the majority of the clones identified in 18-1NT belong instead to the Sphingobacteraceae family (Fig.3).

The partial sequences obtained could be clustered into groups, made up of sequences that consistently branched together in phylogenetic trees and were more than 98% similar. Clones from the 18-NT library amounted to 5 different sequence clusters, while 18-1T partial sequences could be grouped into 3 clusters (Fig. 4 shows the sampling was sufficient). A full length sequence was obtained for each cluster for further analysis. The phylogenetic position of the representative 16S rDNA near full-length sequences in relation to other Alphaproteobacteria, including those previously found to be present in other dinoflagellate cultures, is depicted in Figure 5. The exact phylogenetic association of 18-1T_11B clone could not be supported by our bootstrap analysis. Sequence clusters from 18-1NT were found to be closely associated with bacteria previously identified from dinoflagellate cultures. The cluster of 16S rDNA sequences 18-1NT_10E was placed with bacteria previously isolated from toxic dinoflagellates and found to be associated with *Roseovarius tolerans*. The 18-1NT_12H cluster was found to be closely associated with bacteria previously isolated from *Gymnodinium catenatum* (Green et al, 2004) and placed with the *Sphingomonas*.

The clone library members belonging to the Bacteroidetes could not be further classified through RDP at the pre-determined level of confidence of 95%. The phylogenetic tree (Fig.6) constructed for the Bacteroidetes representative full-length sequences from 18-1T and 18-1NT 16S rDNA clone library show that sequences from each subculture fall mostly in different branches and have different closest relatives in the GenBank database. However, several 16S rDNA clones from both 18-1T and 18-1NT are closely related to isolates of the genus *Microscilla*. The association of 18-1T_11D and 12C and 18-1NT_1E with *Microscilla* is well supported. These were also found to be associated with bacteria isolated from *Gymnodinium catenatum* (Fig. 6).

Mixing experiments

Toxin analysis was performed in cell pellets obtained for late exponential cultures of 18-1T and 18-1NT grown in a chamber that allowed contact through a 5 μm filter for an extended period of time. HPLC analysis showed that the 18-1T subculture was toxic, with peaks identified as GTX1-4. The toxin profile of this 18-1T subculture was very similar to the profile observed for control 18-1T cultures maintained in f/2 medium only, as GTX1 and 4 were the predominant toxins, with lower levels of GTX3 and 2 present. No peaks with retention times corresponding to PSP toxins were observed in chromatograms from 18-1NT extracts.

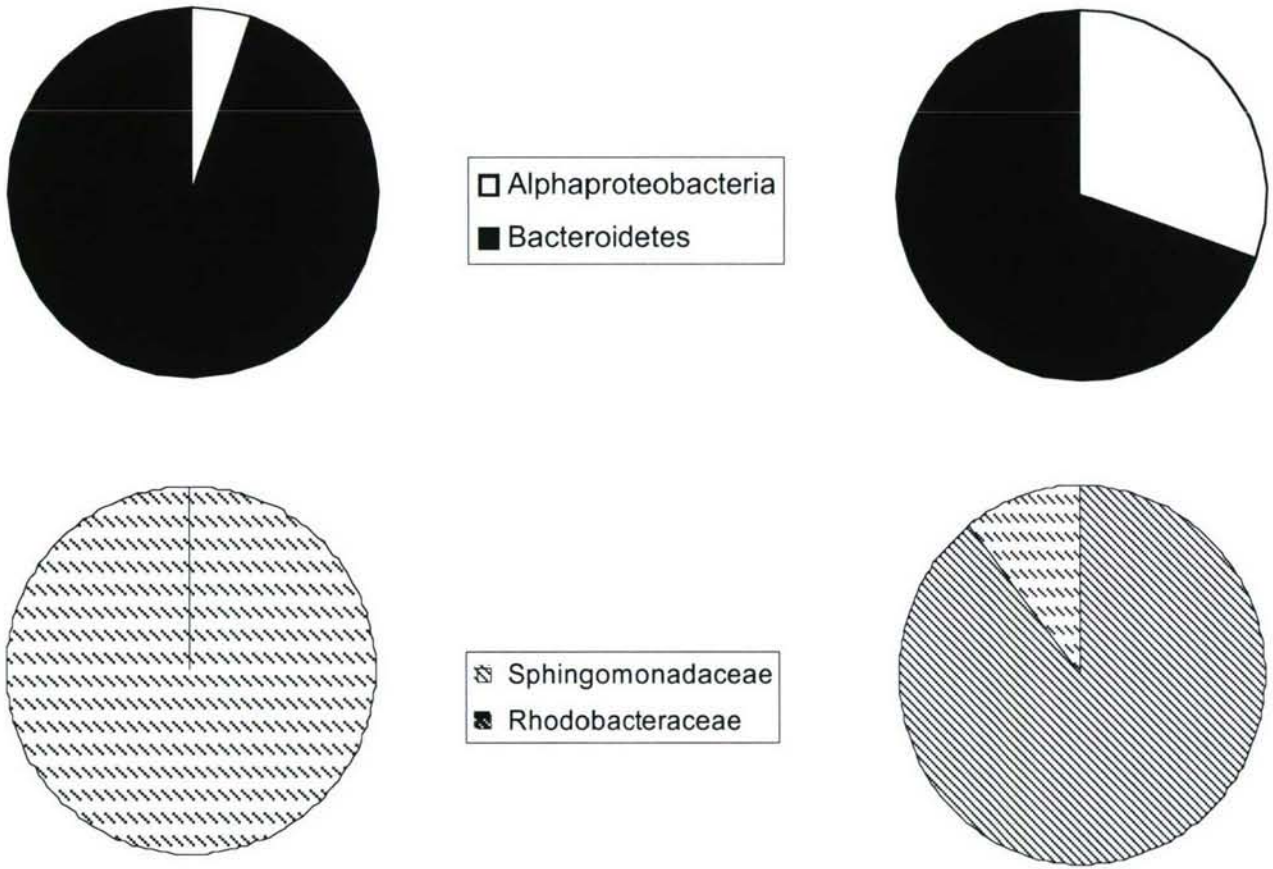

FIGURE 3-3. Proportion of 16s rDNA sequences associated with different Phyla (top) and Family groups within the Alphaproteobacteria (bottom) in cultures of 18-1T (left charts) and 18-1NT (right charts). Associations were determined in the RDP Classifier, using a minimal confidence level of 95%.

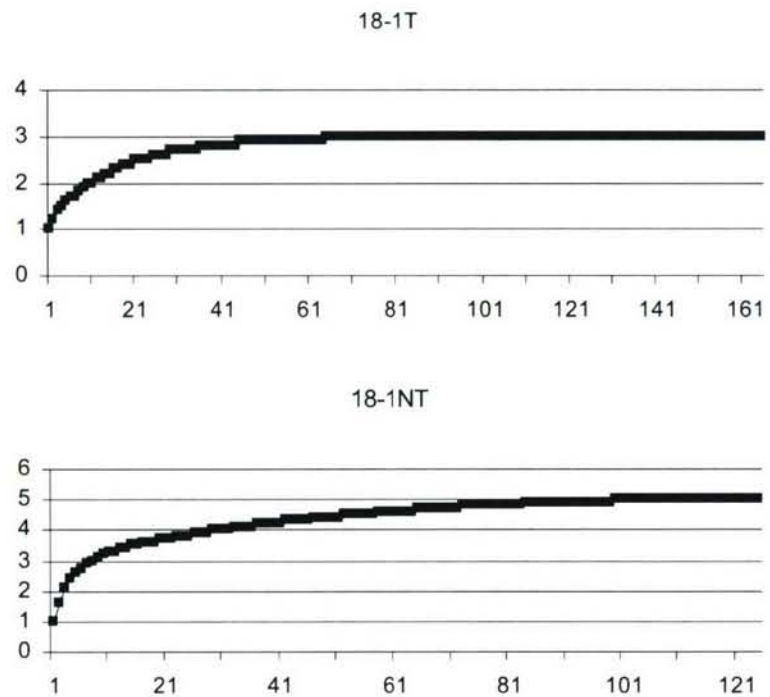

FIGURE 3-4. Rarefaction curves obtained for 18-1T and 18-1NT 16s rDNA clone libraries. Curves were constructed with aRarefactWin.

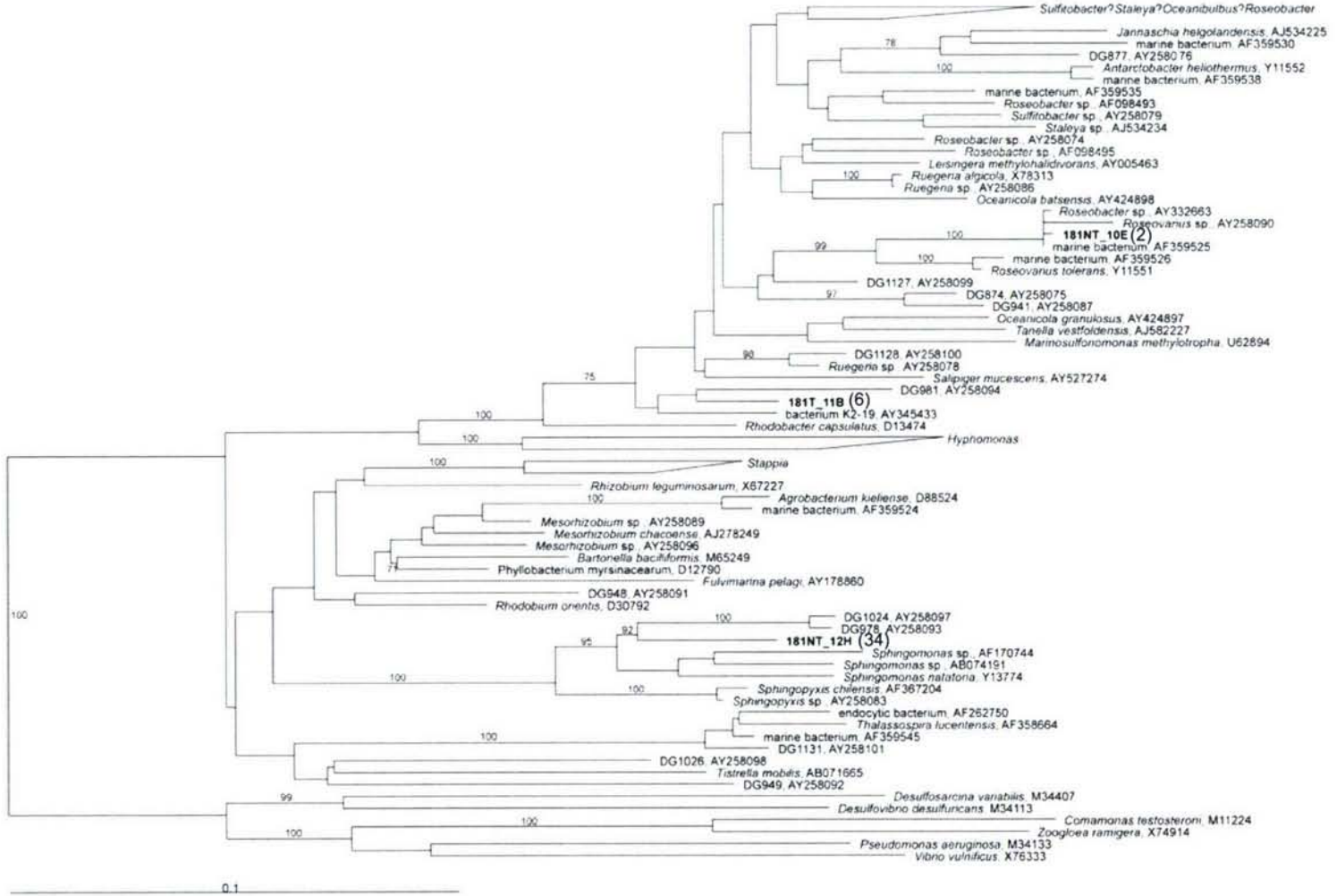

FIGURE 3-5. Phylogenetic analysis of 16s rDNA sequences obtained from cultures of 18-1T and 18-1NT (in boldface type) belonging to the Alphaproteobacteria group. Each sequence shown represents a 98% similarity cluster, comprising of the number of sequences indicated in parenthesis. Tree was constructed by Neighbor joining with Jukes Cantor correction and excluding highly variable regions. Bootstrap support (1000 replicates each) is shown above branches with higher than 70% support. Acession numbers are shown next to sequences.

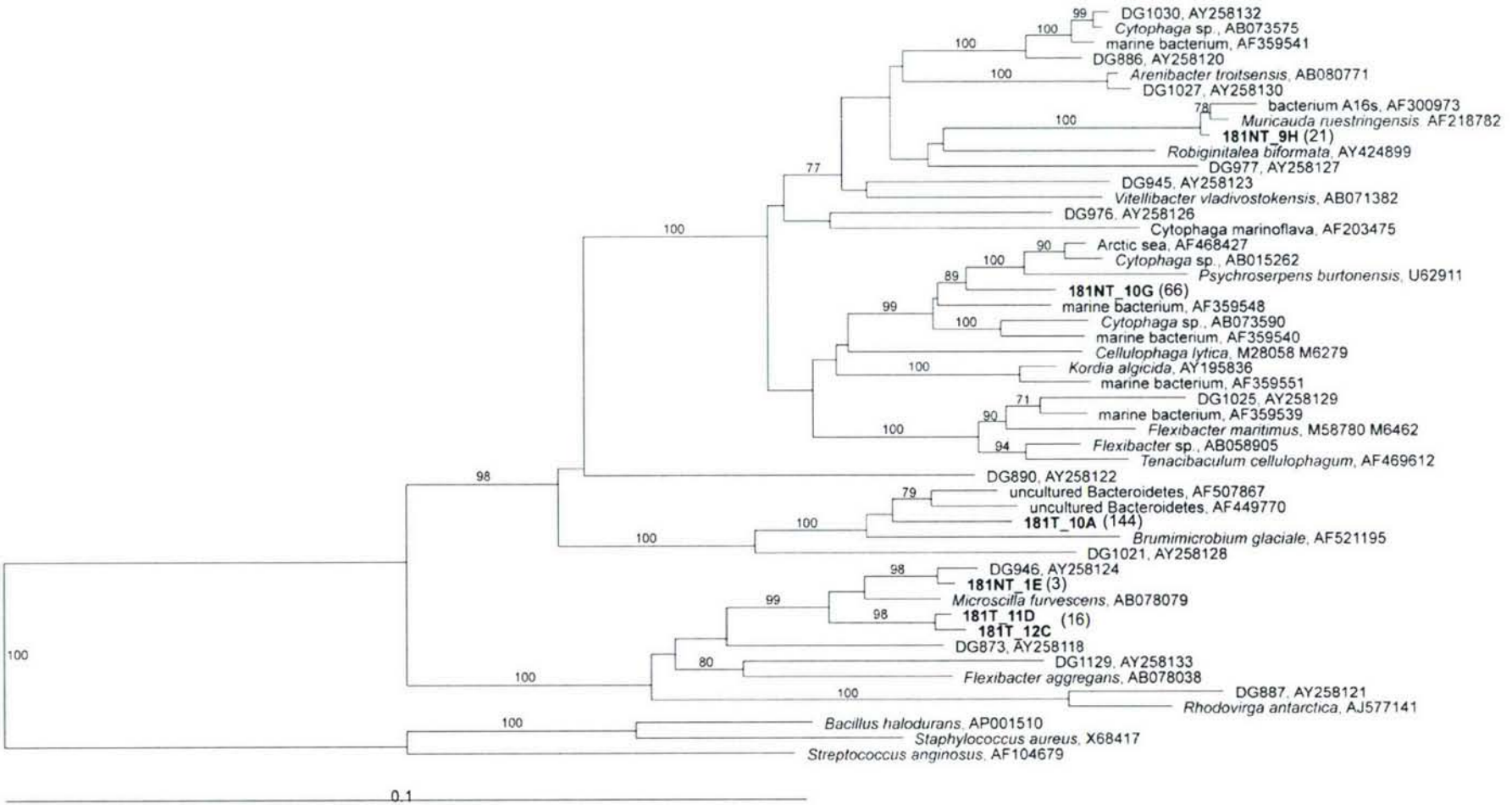

FIGURE 3-6. Phylogenetic analysis of 16s rDNA sequences obtained from cultures of 18-1T and 18-1NT (in boldface type) belonging to the Bacteroidetes group. Each sequence shown represents a 98% similarity cluster, comprising of the number of sequences indicated in parenthesis. Tree was constructed by Neighbor joining with Jukes Cantor correction and excluding highly variable regions. Bootstrap support (1000 replicates each) is shown above branches with higher than 70% support. Acession numbers are shown next to sequences.

Although no alterations in toxin production or toxin profile were observed for 18-1 subcultures grown in the mixing chamber, analysis of the DGGE profile showed that the banding pattern of 18-1T and 18-1NT maintained in contact through a 5 μm filter changed compared to the control cultures. While 18-1T and 18-1NT maintained separately in f/2 medium (Fig. 7 – lanes 1 and 4) show a divergent DGGE banding pattern with no DGGE bands in common, the banding pattern obtained for 18-1 cultures grown in contact consisted of bands shared by both toxic and non-toxic subcultures. This indicates that the growth medium was shared between the two 18-1 subcultures in the mixing chamber and bacteria were able to pass through the 5 μm filter. However, the concurrence of the DGGE banding patterns was not absolute. Band x, (Fig.7) is missing from 18-1T while present in 18-1NT DGGE profile.

Interestingly, the comparison of the associated bacteria of 18-1T and 18-1NT grown in separate culture tubes versus in the mixing chamber revealed that the mixed bacterial assemblage is not a simple addition of the types observed for 18-1T and 18-1NT when grown in isolation.

Cross-inoculation experiments

Prior to cross-inoculation, the bacteria-free status of dinoflagellate cultures was re-checked through 16S rDNA PCR amplification of nucleic acids extracted from the culture. No amplification products could be detected by electrophoresis in an agarose gel, confirming the cultures to be axenic (Fig.8).

After addition of spent medium to the axenic cultures, in vivo fluorescence was recorded and growth curves compared to the axenic and the untreated cultures of toxic and non-toxic 18-1 (Fig.9). Growth rates were calculated for untreated 18-1, axenic 18-1 and 18-1 to which spent media of the other subculture was added (Table 2). 18-1T and 18-1NT subcultures exhibited different growth patterns when compared to each other. Growth rates were similar for all 18-1T cultures grown either in the absence of bacteria or with different bacterial assemblages. However, the axenic cultures could consistently be characterized by a longer lag phase than the xenic counterpart. Similar observations were recorded for 18-1NT (Table 2).

The effect of bacteria on 18-1T and 18-1NT toxicity was studied by analyzing the toxin content of each dinoflagellate culture, at several time points during the growth curve. Cultures were acclimated to the new bacterial community for a minimum of 3 transfers. To guarantee that variation in toxicity due to the growth stage of the culture did not confound results only toxin data obtained when all cultures were in exponential phase were considered (Fig.10). No saxitoxins could be detected by HPLC in any cell extracts of 18-1NT indicating that toxin production was not elicited when spent media of 18-1T was added. 18-1T achieved roughly similar levels of toxicity when maintained in the presence of its own bacterial assemblage or with bacteria previously associated with 18-1NT. However, 18-1T axenic subcultures consistently had lower toxin content compared to bacterized cultures.

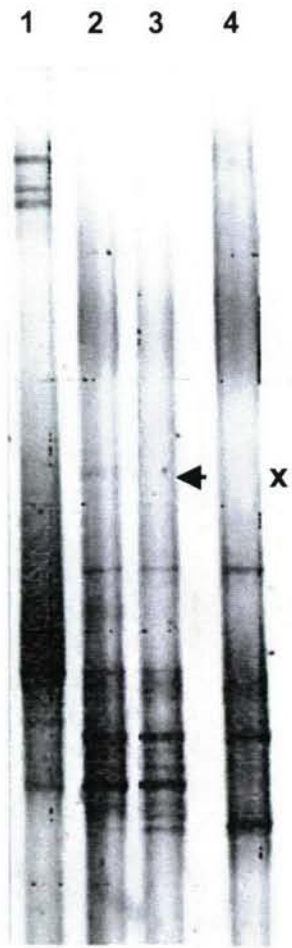

FIGURE 3-7. DGGE patterns of 16s rDNA gene fragments amplified from bacteria present in cultures of 18-1T (2) and 18-1NT (3) grown in contact through a 5 μm filter over a period of 50 days. DGGE patterns of control cultures of 18-1T (1) and 18-1NT (4), grown as isolated cultures, are shown for comparison.

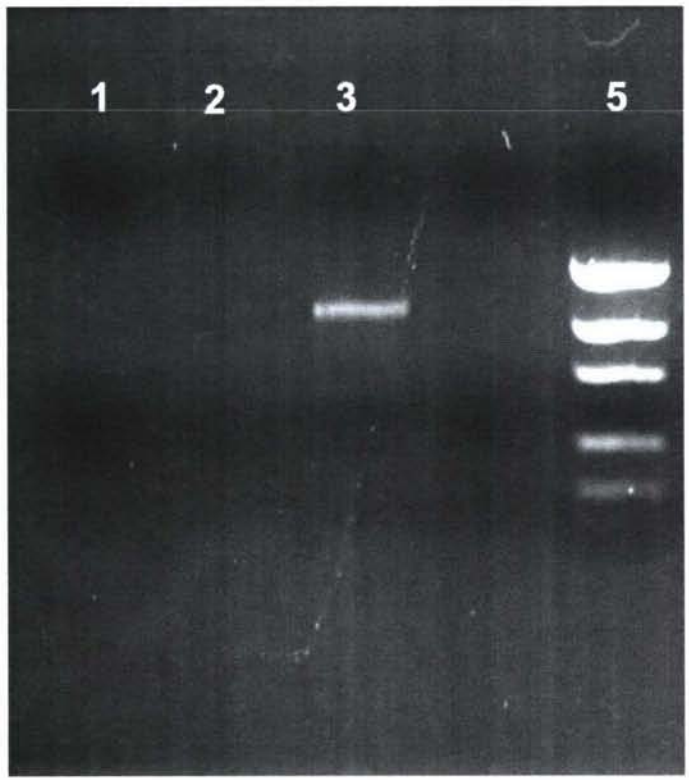

FIGURE 3-8. PCR amplification products obtained from dinoflagellate cultures treated with antibiotics (lane1: 18-1T; lane2: 18-1NT) and control cultures of 18-1 to which no antibiotic was added (lane3). DNA size ladder marker is shown on lane 5. Agarose gel depicted shows products of PCR amplification with UP 16S rDNA primers but is representative of results obtained for several sets of primers (Table1).

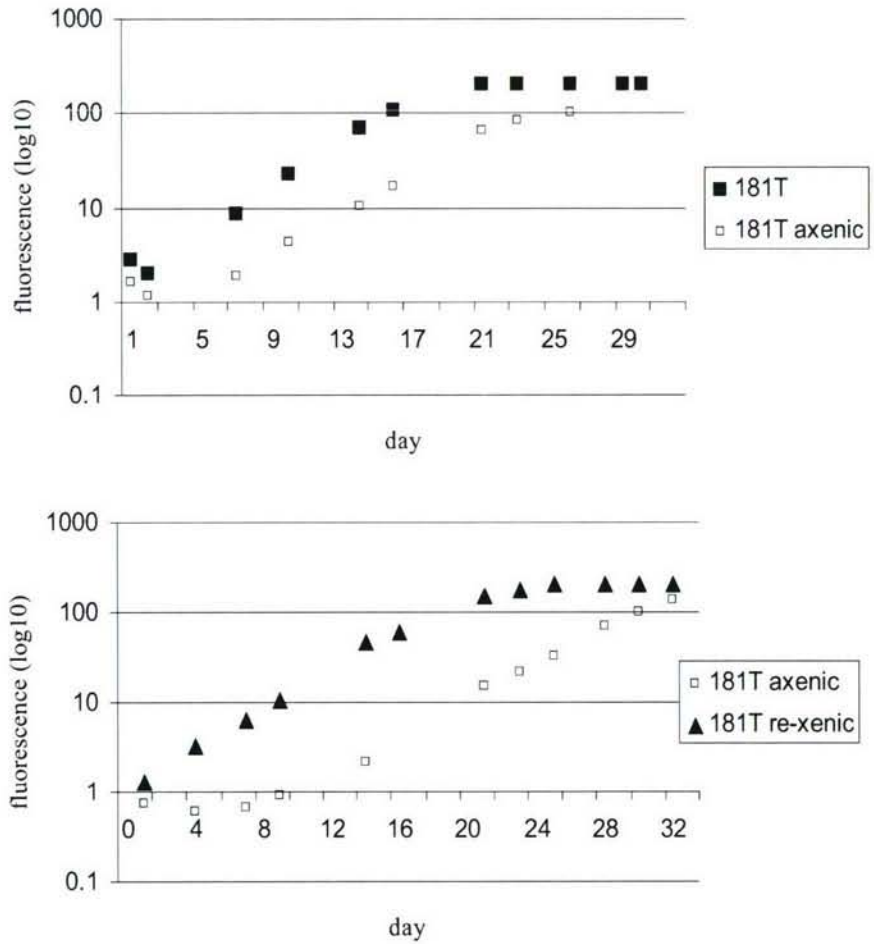

FIGURE 3-9. Growth curves of cultures of 18-1T as determined by fluorescence measurements. Top panel: comparison of growth of control (■) and axenic (□) 18-1T cultures; Bottom panel: comparison of growth curves of axenic cultures (□) and 18-1T axenic culture to which spent medium from the non-toxic 18-1NT culture was added (▲). See Material and Methods for details

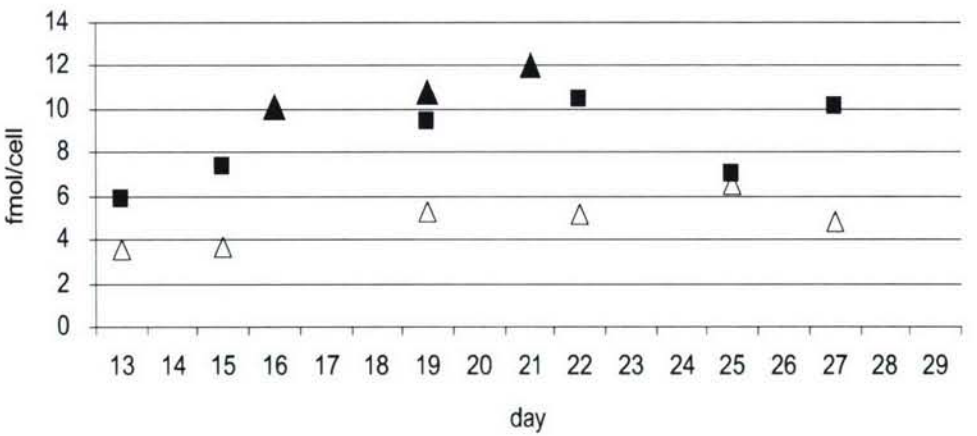

FIGURE 3-10. Toxin content per cell, as determined by HPLC, in control (■) and axenic (Δ) 18-1T dinoflagellate cultures. Toxin content of a 18-1T axenic culture to which spent medium from the non-toxic 18-1NT culture was added is shown in the same graph (▲). Toxin data points are shown for each culture at different time points at exponential growth stage only.

Table 3-2. Growth rate of *A. lusitanicum* subcultures 18-1T and 18-1NT with different bacterial assemblages. Rates are expressed in d^{-1}

Subculture / growth condition	Control[a]	Axenic[b]	Cross-inoculated[c]
18-1T	0.23	0.22	0.23
18-1NT	0.19	0.19	0.20[d]

[a] control cultures were maintained in sterile f/2 growth medium

[b] cultures were rendered axenic following antibiotic treatment. The absence of bacteria was confirmed by the inability to amplify the 16S rDNA gene in 18-1 cultures by PCR (see Material and Methods for details)

[c] cultures were rendered axenic (see above) and subsequently inoculated with spent medium of the other 18-1 culture. All media were filtered through a 2.7 μm filter to exclude whole dinoflagellate cells while allowing bacteria to go through.

[d] Result obtained in a separate experiment

DISCUSSION

The possible role of bacteria in the loss of toxicity and slower growth of clone 18-1NT was tested in this study. Martins et al (2004) hypothesized that the prolonged antibiotic treatment of the parent *A. lusitanicum* culture (18-1) over the years could have eliminated one or more bacterial species necessary for toxin production. However, adding bacteria from the toxic subculture to 18-1NT did not elicit toxin production, nor did it enhance growth of the non-toxic isolate. While bacteria were found to influence growth and toxin production to a certain extent, they do not seem to be the main factor underlying the difference in toxicity in the two subcultures.

Associated bacteria

The study of bacterial assemblages advanced considerably once identification methods were developed that were not dependent on culturing. Numerous molecular approaches have been used to study bacterial-algal interactions. These include construction of clone libraries, DGGE (Schafer et al. 2002) and fluorescent in situ hybridization (FISH) using oligonucleotide probes (Biegala et al. 2002). Since only a small percentage of marine bacteria can be cultured (Schut et al. 1993) the use of molecular techniques to investigate the microorganisms associated with 18-1NT and 18-T is entirely appropriate. In our study, we have employed a combination of the two techniques, DGGE and clone libraries, which will provide information regarding the diversity of bacteria in each culture, as well as their phylogenetic association.

Both methods support the assumption that differences in culture maintenance caused a change in the associated bacteria of 18-1NT. The phylogenetic associations of bacteria from 18-1T and 18-1NT confirmed the divergent DGGE patterns. For example, sequences closely associated with *Sphingomonas* were isolated from the 18-1NT and not from the toxic 18-1T, although both subcultures originated from the same original *A. lusitanicum* 18-1 isolate.

The functional significance of the absence or presence of certain bacterial types in cultures of 18-1T and 18-1NT is thus far unknown. As shown in the phylogenetic analysis in Figures 2 and 3, most 18-1 isolates were closely associated with bacteria previously isolated from other dinoflagellate cultures. However, clear association of bacterial 16s rDNA isolated from 18-1T or 18-1NT with those found in either toxic or non-toxic cultures of dinoflagellates was not obvious from this study. This reflects, in part, the absence of an extensive pool of data to allow such comparisons to be made. Data in GenBank from bacteria associated with dinoflagellates is mostly restricted to cultured bacteria (Hold et al, 2001; Green et al, 2004 and Kopp et al 1997) or shorter sequences (Jasti et al, 2006) that could not be included in our phylogenetic analysis.

Interestingly, this study provides support to the idea that bacteria appear to have a specific association with dinoflagellates, as certain species are frequently isolated from algal cells in cultures and in the field. For example, sequences closely associated with *Microscilla furvescens* were isolated in both 18-1 cultures of *A. lusitanicum* and also

from *G. catenatum* (Green et al, 2004). However, since sequences close to *M. furvescences* were observed in the non-toxic 18-1NT, this particular isolate appears to have no critical role in saxitoxin production.

A further advantage of combining DGGE and 16S rDNA clone library approaches is that while the latter gives a snapshot, DGGE provides a better understanding of the stability of the bacterial assemblage throughout the dinoflagellate growth curve by allowing the comparison of several DGGE profiles in one gel. In this study, 18-1T and 18-1 NT showed highly reproducible DGGE patterns, arguing for the stability of the associated bacteria. Similarly, bacteria cultured from one strain of the dinoflagellate *Ostreopsis* did not change in the course of 48 transfers (Tosteson et al. 1989), while (Alavi et al. 2001) reported the same bacterial assemblage in *Pfiesteria* over a period of two years.

One potential criticism of these conclusions is the possibility of contamination of dinoflagellate cultures with certain bacterial species during prolonged maintenance in laboratory conditions, even though sterile conditions were always used. This could be an alternative explanation for the observed changes in the phylogenetic composition of bacteria associated with 18-1T versus 18-1NT, especially since the cultures were maintained in different laboratories for a period of time and possible contaminants would be of different types. Bacteria introduced when the cultures were kept as separate isolates might not be symbiotic with the dinoflagellate and thus confound the analysis.

Bacterial influence on toxicity

In order to investigate the hypothesis that the different bacterial assemblages caused the loss of toxicity in culture 18-1NT, an approach that bypasses the need to culture bacteria was utilized. The study of direct interactions between dinoflagellates and cultured prokaryotes would be limited since only a small percentage of marine bacteria can be cultured (Schut et al. 1993). Furthermore, culturing bacteria in the absence of the host might affect their phenotypic characteristics and therefore impair identification of symbiotic properties.

Bacterial influence on toxicity was studied by either adding spent dinoflagellate culture medium to axenic toxic and non-toxic dinoflagellate subcultures or by growing the subcultures in a mixing chamber. Both experiments excluded whole dinoflagellate cells while allowing bacteria and metabolites to be exchanged. In addition, both methods allowed physical contact between bacteria and dinoflagellates, found by Doucette and Powell (1998) to be necessary for the microbes to have an effect on toxin production.

Mixing experiments

The possible bacterial influence on 18-1NT or 18-1T toxicity was studied first by growing both cultures in a mixing chamber. In these growth conditions, culture media and particles smaller than 5 μm could be freely exchanged between the two 18-1 subcultures. The mixing of the bacterial assemblages was confirmed by the highly similar DGGE patterns of cultures in the mixing chamber. Despite the convergence of associated

bacteria, the toxicity of 18-1T and 18-1NT was not different from control cultures grown separately. While this seems to indicate that bacteria did not affect toxin production, it should be noted that the convergence of bacterial assemblages was not complete. Indeed bacteria attached to the dinoflagellate are unlikely to have migrated through the chamber and it is a possible explanation for the incomplete convergence of the bacterial assemblages. Furthermore, the process might take more time than was allowed in these experiments (50 days). However, results from this experiment should not be judged separately, but in conjunction with further results detailed below.

In view of these results, the study by Sousa Silva (1990) remains the only report of bacteria, previously isolated from a toxic dinoflagellate culture, being capable of eliciting the production of some toxicity when inoculated in a non-toxic dinoflagellate clone. Since Sousa Silva's toxin measurements were obtained by the less sensitive mouse bioassay, it is possible that the addition of bacteria increased previously undetectable saxitoxin concentrations in *Prorocentrum minimum*. In other words, some toxin production capability may have been present, which was then enhanced by the bacterial addition. It is also possible that a compound other than saxitoxin was responsible for the mouse deaths, given the non-specificity of that bioassay.

Surprisingly, the mixed bacterial assemblage of 18-1T and 18-1NT differed somewhat from the original 18-1T and 18-1NT cultures grown separately, rather than being a simple mix of the two. The DGGE profiles seem to indicate that the composition of the bacterial assemblage of a dinoflagellate might be dynamic and depend on interaction between the bacterial species present. To our knowledge, bacteria/bacteria interactions and how this might affect the final composition of the bacterial assemblage of a dinoflagellate either in the field or in culture has not been addressed previously.

Axenic cultures

The effect of removing bacteria on the toxin content of 18-1T was recorded as part of our experiment. A few reports are available addressing the effects of removing bacteria on dinoflagellate toxicity. However, the axenic status of the dinoflagellate culture in these studies is sometimes doubtful, as the presence or absence of bacteria was ascertained by inoculation of cultures into different bacterial growth media (Dantzer and Levin 1997) or by the microscopic observation of acridine orange stained samples (Doucette and Powell 1998). In this study we have chosen PCR as a means to detect bacterial 16S rDNA. This technique can detect the presence of both non-culturable and intra-cellular bacteria and has a reported limit of detection of 10 cfu in a PCR reaction, although 1 cfu can occasionally be amplified (Hold et al 2001). We have restricted the comparison of our study to others using a similar method to demonstrate the axenic status of cultures.

Consistent with results reported by Uribe and Espejo (2003) for *Alexandrium catenella*, this study demonstrated that bacteria did influence toxin production by the dinoflagellate, as cultures deemed axenic had a lower toxin content when compared to untreated xenic, cultures. One common criticism of the Uribe and Espejo (2003) study, as well as of a similar report by Hold et al (2001), is that the lower toxin content of axenic cultures

resulted from the antibiotic treatment, rather than the loss of bacteria. Here we demonstrated that the differences in the toxin content of axenic cultures were not due to the antibiotic treatment, as re-addition of bacteria restored toxicity to levels previously recorded (see below).

As described in previous reports, results show axenic dinoflagellate cultures continue to produce saxitoxins, despite alterations of the toxin content. There are no reports that describe the total loss of saxitoxin production when cultures are made axenic. In view of these findings, it seems unlikely that the loss of toxicity by 18-1NT could be attributed to the loss of bacteria during culture maintenance with antibiotic treatment.

Cross-inoculation experiments

The effect of a specific bacterial assemblage in dinoflagellate toxicity was investigated by removing bacteria from one subculture and replacing them with the bacterial assemblage of the other. Results show the toxin levels observed for axenic 18-1T after re-introduction of bacteria were similar to those of untreated 18-1T cultures, despite the fact that bacteria re-introduced were previously associated with a non-toxic 18-1NT culture.

Similarly, in cultures of *Pseudo-nitzchia,* the introduction of bacteria into axenic cultures did increase the production of domoic acid (Bates et al. 1995). Bacteria shown to cause an increase in toxin content had been isolated from *Pseudo-nitzchia,* but also from the non-toxic diatom *Chaetoceros*. These results indicate that several types of bacteria might be capable of affecting the toxin content of algal cultures, whether or not those bacteria originated from toxic or non-toxic isolates. No further information is available on specificity of the interaction and whether bacteria typically not associated with algae could produce the same effect. Since these are the only studies available that address the contribution of bacteria to toxin production by re-inoculating bacteria in axenic cultures it is difficult to identify a general relationship.

Adding bacteria from 18-1T to axenic 18-1NT did not elicit toxin production in the non-toxic subculture. Though it is possible the transfer of bacteria from 18-1T was not complete, this and previously discussed observations seem to indicate that changes in the bacterial assemblage did not cause the loss of toxicity in 18-1NT. Furthermore, the fact that subcultures were maintained with the inoculated bacteria for at least 3 transfers before experiments began allowed for sufficient time for the bacteria to influence the dinoflagellate metabolism.

Bacteria influence on growth

The relationship between bacteria and dinoflagellates is complex. In natural populations, bacteria are known to both stimulate and inhibit dinoflagellate growth. Bacterial populations associated with early stage blooms of dinoflagellate are shown to increase the growth rate of dinoflagellate cultures, while bacteria associated with late stage blooms inhibit dinoflagellate growth (Doucette, 1995). In culture, the effect of bacteria on dinoflagellate growth is poorly studied, as most attention has been focused on the effect

of bacteria on dinoflagellate toxin production. In the present study we have tried to address the effect of bacteria in dinoflagellate growth by directly comparing the growth of untreated, axenic and cross-inoculated cultures of 18-1T and 18-1NT.

Our results indicate that while bacteria do not seem to influence growth rate of *A. lusitanicum*, they do have an effect on the duration of the lag phase. Interestingly, (Hold et al. 2001) report very similar findings, as non-axenic *A. lusitanicum* showed a higher rate of increase of cell concentration in the first 8 days of culturing compared to the axenic counterpart. The presence of bacteria could decrease the length of the lag phase of growth, as these microorganisms might convey certain nutrients essential for initial phases of growth, as suggested by (Doucette 1995). Furthermore, bacteria could secrete signaling and communication molecules influencing dinoflagellate metabolism (Vardi et al. 2002).

It has been hypothesized that the use of an antibiotic cocktail could be responsible for the differences between the growth of axenic and non-axenic cultures. The effect of antibiotics on dinoflagellate growth is unknown but not unlikely. Known effects of antibiotics in some algal species include the sustained loss of pigmentation (Dube 1952), changes in chloroplast morphology and reduction in growth (Miyoshi and Tsubo 1969). These effects seem to remain even after multiple transfers in antibiotic-free culture medium (Provasoli et al. 1948).

The effect of antibiotics on dinoflagellate growth was directly addressed in our experimental design. If we assume the removal of the bacteria and not the antibiotic treatment was responsible for the differences in growth then adding back the bacteria to an antibiotic treated culture should restore the growth patterns observed prior to the antibiotic addition. Our results demonstrate that adding bacteria in the form of dinoflagellate spent media restores the growth patterns to rates seen in cultures not treated with antibiotics.

However, while growth was restored to the levels seen in non-axenic cultures, adding bacteria from 18-1T to 18-1NT did not lead to an increase of the growth rate and maximum cell density of 18-1NT to the levels characteristic of 18-1T. Similarly, growth rate and cell yield of 18-1T plus 18-1NT bacteria were identical to untreated cultures of 18-1T rather than 18-1NT. In other words, 18-1 maximum cell yield and division rate seem to depend on the dinoflagellate strain and growth conditions, rather than the bacterial assemblage present. We have tried to address the issue of whether bacteria or metabolites provided in the spent media are responsible for the differences in growth. However, cultures inoculated with spent media filtered through a 0.2 μm filter could not be shown to be bacteria free.

Similar to findings of the previous section, the reduction in growth observed in the 18-1NT when compared to 18-1T could not be attributed to the different bacterial assemblages of the 18-1 subcultures. However, bacteria do affect dinoflagellate growth per se, by decreasing the length of the lag phase of growth. This interaction, if proven in field populations, could have implications for bloom dynamics.

CONCLUSIONS

No saxitoxins could be detected in cultures of 18-1NT, independently of the presence, absence or composition of associated bacteria. We were unable to elicit toxin production in 18-1NT even when bacteria from the toxic, faster growing 18-1T culture were added alone or in combination with the 18-1NT assemblage. Both the reduction in growth rate and the loss of toxicity in 18-1NT seems to be intrinsic to the culture and be independent of the bacterial assemblage.

Our initial hypothesis that the antibiotic treatment to which 18-1NT had been subjected caused a change in the bacterial assemblage seems valid in view of the phylogenetic analysis and DGGE data. However, it seems unlikely that the change in associated bacteria caused the reduction in growth and loss of toxicity in 18-1NT. Despite the caveats noted for each of the experiments discussed, all experiments are in agreement that bacteria did not play a role in changes observed in 18-1NT.

The data presented supports the view that toxin synthesis is most likely coded by dinoflagellate chromosomal genes, as some studies seem to indicate (Sako et al. 1995). In the case of the 18-1NT subculture, it is possible that regions of the genome could have suffered and accumulated mutations over the time period when the cultures were kept as individual isolates. Such mutations could have affected a gene or genes in the saxitoxin biosynthetic pathway and /or hinder growth. The functional genomics of 18-1T and 18-1NT is currently being studied, as it is assumed it will reflect the loss of toxin production of the subculture, as well as the observed differences in growth capacity.

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REFERENCES

Alavi, M., T. Miller, K. Erlandson, R. Schneider, and R. Belas. 2001. Bacterial community associated with *Pfiesteria*-like dinoflagellate cultures. Environmental Microbiology **3:** 380-396.

Alverca, E., I. Biegala, G. Kennaway, J. Lewis, and S. Franca. 2002. In situ identification and localization of bacteria associated with *Gyrodinium instriatum* (Gymnodiniales, Dinophyceae) by electron and confocal microscopy. European Journal of Phycology **37:** 523-530.

Anderson, D. M., D. M. Kulis, G. J. Doucette, J. C. Gallagher, and E. Balech. 1994. Biogeography of toxic dinoflagellates in the genus *Alexandrium* from the northeastern United States and Canada. Marine Biology **120:** 467-478.

Baker, T. R., G. J. Doucette, C. Powell, G. L. Boyer, and F. G. Plumley. 2003. GTX4 imposters: characterization of fluorescent compounds synthesized by *Pseudomonas stutzeri* SF / PS and *Pseudomonas* / *Alteromonas* PTB-1, symbionts of saxitoxin-producing *Alexandrium* spp. Toxicon **41:** 339-347.

Bates, S. S., D. J. Douglas, G. J. Doucette, and C. Leger. 1995. Effects of reintroducing bacteria on domoic acid production by axenic cultures of the diatom *Pseudo-nitzschia pungens* f. multiseries, p. 401-406. *In* P. Lassus, G. Arzul, E. Erard-Le Denn, P. Gentien and C. Marcaillou-Le Baut [eds.], Harmful Marine Algal Blooms. Proceeding of the Sixth International Conference on Toxic Marine Phytoplankton, Nantes (France), Oct 1993. Lavoisier.

Biegala, I., G. Kennaway, E. Alverca, J.-F. Lennon, D. Vaulot, and N. Simon. 2002. Identification of bacteria associated with dinoflagellates (Dinophyceae) *Alexandrium* spp. using tyramide signal amplification - fluorescent *in situ* hybridization and confocal microscopy. Journal of Phycology **38:** 404-411.

Buchholz-Cleven, B., B. Rattunde, and K. Straub. 1997. Screening for genetic diversity of isolates of anaerobic Fe(II)-oxidising bacteria using DGGE and whole cell hybridization. Systematic Applied Microbiology **20:** 301-309.

Cembella, A. D. 1998. Ecophysiology and metabolism of paralytic shellfish toxins in marine microalgae, p. 381-403. *In* D. M. Anderson, A. D. Cembella and G. M. Hallegraeff [eds.], Physiological Ecology of Harmful Algal Blooms. NATO ASI Series. Springer-Verlag.

Dantzer, W. R., and R. E. Levin. 1997. Bacterial influence on the production of paralytic shellfish toxins by dinoflagellate algae. Journal of Applied Microbiology **83:** 464-469.

Doucette, G. J. 1995. Interactions between bacteria and harmful algae: A review. Natural Toxins **3:** 65-74.

Doucette, G. J., and C. Powell. 1998. Algal-bacteria interactions: can they determine the PSP-related toxicity of dinoflagellates, p. 406-409. *In* B. Reguera, Blanco, J., Fernandez, M.L. and Wyatt, T. [ed.], Harmful algae. Xunta de Galicia and IOC.

Doyle, J. J., and J. L. Doyle. 1990. Isolation of plant DNA from fresh tissue. Focus **12:** 13-15.

Dube, J. F. 1952. Observation on chlorophyll deficient strain of *Chlorella vulgaris* obtained after treatment with streptomycin. Science **116:** 278.

Franca, S. and others 1995. Prokaryotes in association with a toxic *Alexandrium lusitanicum* in culture, p. 45-51. *In* P. Gentien and C. Marcaillou-Le Baut [eds.], Harmful Marine Algal Blooms/Proliferation d'Algues Marines Nuisibles. Proceedings of the Sixth International Conference on Toxic Marine Phytoplankton, Nantes (France), Oct 1993. Lavoisier.

Gallacher, S., and E. Smith. 1999. Bacteria and Paralytic Shellfish Toxins. Protist **150:** 245-255.

Green, D. H., L. Llewellyn, A. Negri, S. I. Blackburn, and C. Bolch. 2004. Phylogenetic and functional diversity of the cultivable bacterial community associated with the

paralytic shellfish poisoning dinoflagellate *Gymnodinium catenatum*. FEMS Microbiology Ecology **47:** 345-357.

Guillard, R. R. L., and J. H. Ryther. 1962. Studies of marine plankton diatoms I. *Cyclotella nana* Hustedt and *Detonula confervacea* (Cleve) Gran. Canadian Journal of Microbiology **8:** 229-239.

Hold, G. L., E. A. Smith, T. H. Birkbeck, and S. Gallacher. 2001. Comparison of paralytic shellfish toxin (PST) production by the dinoflagellates *Alexandrium lusitanicum* NEPCC 253 and *Alexandrium tamarense* NEPCC 407 in the presence and absence of bacteria. FEMS Microbiology Ecology **36:** 223-234.

Hugenholtz, P., and T. Huber. 2003. Chimeric 16S rDNA sequences of diverse origin are accumulating in the public databases. International Journal of Systematic Evolutionary Microbiology **53:** 289-293.

Humpage, A. R. and others 1994. Paralytic shellfish poisons from Australian cyanobacterial blooms. Australian Journal of Marine and Freshwater Research **45:** 761-771.

Jasti, S., M. E. Sieracki, N. J. Poulton, M. W. Giewat, and J. N. Rooney-Varga. 2005. Phylogenetic diversity and specificity of bacteria closely associated with *Alexandrium* spp. and other phytoplankton. Applied and Environmental Microbiology **71:** 3483-3494.

Lafay, B., R. Ruimy, C. Rausch de Traubenberg, V. Breittmayer, M. J. Gauthier, and R. Christen. 1995. *Roseobacter algicola* sp. nov., a new marine bacterium isolated from the phycosphere of the toxin-producing dinoflagellate *Prorocentrum lima*. International Journal of Systematic Bacteriology **45:** 290-296.

Ludwig, W. and others 2004. ARB: a software environment for sequence data. Nucleic Acids Research **32:** 1363-1371.

Maidak, B., G. Olsen, N. Larsen, R. Overbeek, M. McCaughey, and C. Woese. 1999. The RDP (Ribosomal database Project). Nucleic Acids Research **25:** 109-110.

Martins, C. A., P. Alvito, M. J. Tavares, P. Pereira, G. Doucette, and S. Franca. 2003. Reevaluation of production of paralytic shellfish toxin by bacteria associated with dinoflagellates of the Portuguese coast. Applied and Environmental Microbiology **69:** 5693-5698.

Martins, C. A., D. Kulis, S. Franca, and D. M. Anderson. 2004. The loss of PSP toxin production in a formerly toxic *Alexandrium lusitanicum* clone. Toxicon **43:** 195-205.

Miyoshi, Y., and Y. Tsubo. 1969. Permanent bleaching of *Euglena* by chloramphenicol. Plant & Cell Physiology **10:** 221-225.

Oshima, Y. 1995. Post-column derivatization HPLC methods for paralytic shellfish poisons, p. 81-94. *In* G. M. Hallegraeff, D. M. Anderson and A. D. Cembella [eds.], Manual on harmful marine microalgae, IOC manuals and guides. UNESCO.

Posada, D., and K. Crandall. 1998. Modeltest: testing the model of DNA substitution. Bioinformatics **14:** 817-818.

Provasoli, L. 1963. Growing marine seaweeds, p. 9-17, 4th International Seaweed Symposium.

Provasoli, L., S. Hutner, and A. Schatz. 1948. Streptomycin-induced chlorophyll-less races of *Euglena*. Proc. Soc. Exp. Biol. and Med. **69:** 279-282.

Sako, Y., N. Naya, T. Yoshida, C.-H. Uchida, and Y. Ishida. 1995. Studies on stability and heredity of PSP toxin composition in the toxic dinoflagellate *Alexandrium*., p. 345-350. *In* P. Lassus, G. Arzul, E. Erard, P. Gentien and C. Marcaillou [eds.], Harmful Marine Algal Blooms. Lavoisier.

Schafer, H., B. Abbas, H. Witte, and G. Muyzer. 2002. Genetic diversity of 'satellite' bacteria present in cultures of marine diatoms. FEMS Microbiology Ecology **42:** 25-35.

Schafer, H., and G. Muyzer. 2001. Denaturing gradient gel electrophoresis in marine microbial ecology, p. 425-468. *In* J. Paul [ed.], Methods in Microbiology. Academic Press.

Schut, F. and others 1993. Isolation of typical marine bacteria by dilution culture: growth, maintenance, and characteristics of isolates under laboratory conditions. Applied and Environmental Microbiology **59:** 2150-2160.

Silva, E. S. 1962. Some observations on marine dinoflagellate cultures. I. *Prorocentrum micans* Ehr., *Gymnodinium spinifera* (Clap. and Lach.) dies., *Gonyaulax tamarensis* Leb., and *Peridinium trochoideum* (Stein) Lemm. Notas e Estudos do Instituto de Biologia Marinha **26:** 1-21.

Silva, E. S., and I. Sousa. 1981. Experimental work on the dinoflagellate toxin production. Separata dos Arquivos do INSA **VI:** 381-387.

Sousa Silva, E. 1990. Intracellular bacteria: the origin of dinoflagellate toxicity. Journal of Environmental Pathology, Toxicology, and Oncology **10:** 124-128.

Swofford, D. 2001. PAUP: Phylogenetic analysis using parsimony. Sinauer Associates.

Tosteson, T. R., D. L. Ballantine, C. G. Tosteson, V. Hensley, and A. T. Bardales. 1989. Associated bacterial flora, growth, and toxicity of cultured benthic dinoflagellates *Ostreopsis lenticularis* and *Gambierdiscus toxicus*. Applied and Environmental Microbiology **55:** 137-141.

Uribe, P., and R. T. Espejo. 2003. Effect of associated bacteria on the growth and toxicity of *Alexandrium catenella*. Applied and Environmental Microbiology **69:** 659-662.

Utermöhl, H. 1958. Neue Wege in der quantitativen Erfassung des Planktons (mit besonderer Berücksichtigung des Ultraplanktons. Verh. int. Ver. theor. angew. Limnol. **5:** 567-596.

Vardi, A. and others 2002. Dinoflagellate-cyanobacterium communication may determine the composition of phytoplankton assemblage in a mesotrophic lake. Current Biology **12:** 1767-1772.

Weisburg, W., M. Barns, D. Pelletier, and D. Lane. 1991. 16S ribosomal DNA amplification for phylogenetic study. Journal of Bacteriology **173:** 697-703.

CHAPTER 4

PROTEOMIC ANALYSIS OF A NON - TOXIC SUBCULTURE OF *ALEXANDRIUM LUSITANICUM*

ABSTRACT

Paralytic Shellfish Poisoning (PSP) is a potentially life-threatening condition caused by the consumption of bivalves contaminated by a family of neurotoxins called saxitoxins. While the chemistry and effects of these toxins are fairly well understood, the biochemical pathway and consequently the genes and enzymes involved in saxitoxin production are still poorly characterized in dinoflagellates. In this study, a culture of *Alexandrium lusitanicum* that is defective in toxin production and growth capability was investigated. A toxic faster growing subculture of the same isolate also exists, together constituting a novel experimental system to study gene expression patterns related to toxin production and growth.

The proteome of this pair of subcultures was analyzed by two-dimensional difference gel electrophoresis (DIGE) and two-dimensional gel electrophoresis with unique proteins identified by peptide mass fingerprinting. The results demonstrate that DIGE is a viable technique for the comparative study of dinoflagellates. Furthermore, it allowed for the identification of proteins differentially expressed between the toxin-deficient mutant and the wild type. Identification of some proteins of interest was possible by searching the Expressed Sequence Tag (EST) database for dinoflagellates, demonstrating that dinoflagellate proteins can be identified through mass spectrometry, even though a full genome sequence or extensive protein database is not available for these organisms. Proteins that could be identified as down regulated in the non-toxic, slower-growing subculture are all enzymes from the Calvin cycle. The reduction in this cycle may explain the poor growth of the non-toxic isolate, as cells are thought to be limited in their ability to utilize carbon dioxide. Other unknown, differentially expressed proteins may relate to the loss of toxicity, but their identity and function remain unresolved.

INTRODUCTION

Paralytic Shellfish Poisoning (PSP) is a potentially life threatening condition caused by the consumption of bivalves contaminated by a family of neurotoxins called saxitoxins. Due to the broad global distribution of PSP and the severity of symptoms, PSP is arguably the most significant of the human poisoning syndromes linked to the proliferation of harmful algal blooms. The dinoflagellate genus *Alexandrium* comprises a considerable number of species responsible for the production of saxitoxins involved in PSP (Taylor and Fukuyo 1998) and has a worldwide distribution. Other dinoflagellates and freshwater cyanobacteria have also been shown to produce these toxins. While the

chemistry and mechanisms of action of saxitoxin are fairly well characterized, the biosynthetic pathway (Shimizu et al. 1984) and consequently, the physical and biological factors that affect the severity of PSP episodes, remain poorly understood.

Since the first studies by Shimizu (eg. (Shimizu et al. 1984), little progress has been made in identifying the multiple enzymes (Cembella 1998) and genes that are presumably involved in the synthesis of the parent molecule (Plumley 1997). In light of the pathway proposed by the study of Shimizu, very recently (Kellman and Nielan 2006 (oral presentation)) have identified potential genes and intermediates involved in the saxitoxin biosynthetic pathway in toxic cyanobacteria. It remains unclear how this knowledge can be applied to dinoflagellates.

The description of genes and enzymes involved in toxin production has in part been hindered by the inability to create and identify toxin-deficient mutant cells (Plumley 2001). Attempts were made to circumvent this issue by studying genetically related *Alexandrium* strains that differ in their ability to produce toxins (D. Erdner, umpublished data). This approach has been largely unsuccessful presumably due to DNA sequence differences between the toxic strains and the closely related non-toxic strains to which they are compared. Naturally occurring toxic and non-toxic cells may be the same morphospecies but considerable genetic differences have been noted in genes that presumably have no direct influence on whether a species does or not produce a toxin (eg. ribosomal RNA, (Scholin et al. 1994). Comparing cells grown under nitrate and phosphate starvation, conditions that respectively decrease and increase toxin (Anderson 1991), has been another experimental approach to identify genes involved in toxin production (D. Erdner, unpublished data). However, results will most likely reflect the incomplete suppression of toxin production under nitrate limited conditions. In addition, genes not necessarily involved in toxin production, but rather associated with nutrient stress, are also differentially expressed and will be identified.

The use of conventional mutational analysis or transformation to create toxin-deficient dinoflagellate cells is constrained by biological and technical factors associated with these organisms. These include widespread gene duplication involving many gene copies (Erdner and Anderson 2006; Le et al. 1997; Lee et al. 1993) and the logistical challenge of screening thousands of candidates for toxicity to detect those cultures in which a toxin gene has been inactivated. However, a spontaneous mutant of *Alexandrium lusitanicum* that has lost the ability to produce saxitoxins was recently identified (Martins et al. 2004). The original *A. lusitanicum* culture was isolated from a toxic bloom and shown to be toxic by the mouse bioassay (MBA) and subsequently by high performance liquid chromatography (HPLC) (Cembella 1988; Mascarenhas et al. 1995). Two subcultures of this isolate were established and maintained as separate isolates. Three independent toxin analysis methods (MBA, HPLC and mouse neuroblastoma assay) showed that while one of the subcultures maintained the same levels of toxicity and the toxin profile as the initial culture, the other no longer produced detectable toxin levels. To distinguish subcultures producing saxitoxin from those lacking toxicity, subcultures were re-named 18-1T and 18-1NT, respectively. Further observation revealed differences in growth rates

and maximum cell density between the two subcultures, as 18-1NT was consistently slower growing than the toxic variant (Martins et al, 2004).

Previous work (Chapter 3) has demonstrated that the loss of toxicity of 18-1NT is intrinsic to the culture and not dependent on the bacterial symbionts present. We can therefore work from the hypothesis that gene and/or protein expression have been altered in the 18-1NT strain, presumably due to a mutation which would have occurred while the original isolate was maintained as two separate cultures. If this is the case, gene expression and gene products of toxic and non-toxic *A. lusitanicum* would most likely reflect the loss of toxicity in the 18-1NT subculture, as well as the observed differences in growth. Because the two 18-1 cultures are very closely related and are maintained in similar growth conditions, the remaining genes expressed in each and their products should be highly shared.

This pair of cultures is an ideal experimental system with which to study the mechanisms potentially involved in toxin production and dinoflagellate growth. Several techniques are available to compare the gene expression of 18-1T versus 18-1NT. Methods such as differential display (Taroncher-Oldenburg and Anderson 2000) and MPSS (Erdner and Anderson, 2006) have been previously successfully applied to the study of transcriptionally regulated genes of dinoflagellates of the genus *Alexandrium*. However, since dinoflagellates employ both transcriptional and post-transcriptional gene regulation (Erdner and Anderson 2006; Fagan et al. 1999; Taroncher-Oldenburg and Anderson 2000), a more comprehensive characterization of mutants and comparison with the wild-type counterpart can be obtained by the study of the proteome. The applicability of proteomic analysis to dinoflagellates has recently been demonstrated by Chan and co-workers (Chan et al. 2002), using two-dimensional gel electrophoresis (2-D) analysis for dinoflagellate species recognition (Chan et al. 2004) and to detect biomarkers of toxicity (Chan et al. 2005b).

The use of comparative proteomic analyses, known as functional proteomics, will be particularly pertinent to our experimental model. Functional proteomics has successfully identified disease-specific proteins or proteins related to gene knock-outs in various organisms (Graves and Haystead 2002). Difference gel electrophoresis (DIGE) in particular is an ideal method for differential analysis. Protein samples are labeled individually with one of two fluorescent dyes, and then mixed and resolved by two-dimensional gel electrophoresis. Differences in expression are visualized and detected by merging the fluorescent gel images obtained at two different wavelengths. Because DIGE allows two protein samples to be run in the same gel it reduces the gel-to-gel variation observed during separate 2-D runs. Changes in protein abundance between 18-1T and 18-1NT should then be identifiable.

Proteins that were not shared between toxic and non-toxic 18-1 strains were given in-depth attention as they might play a role in the saxitoxin pathway or in the observed changes in growth between the two cultures. The suitability of DIGE to the study of differentially expressed dinoflagellate proteins was established and extraction and separation methods were optimized. Proteins of interest, identified through DIGE, were

further studied by two-dimensional electrophoresis and identification attempted through peptide mass fingerprinting.

MATERIAL AND METHODS

Dinoflagellate cultures

The original 18-1 *A. lusitanicum* culture was isolated from a bloom in Lagoa de Obidos, Portugal in 1962. In 1992 this isolate was divided into two subcultures: one was maintained in Dr. S Franca's laboratory (Portugal) in ASP7 with the periodic addition of the AM9 antibiotic mixture (Provasoli 1963), while the other was sent to Dr. G. Doucette's laboratory (USA) and transferred to f/2 medium, without antibiotics. These subcultures were shown to differ in toxicity in 2000, upon which they were renamed 18-1NT (non-toxic) and 18-1T (toxic), respectively, and transferred to identical growth conditions. For protein analysis, all cultures were maintained in sterile f/2 growth medium (Guillard 1975) at 21°C on a 16:8 light:dark cycle with a fluorescent illumination of approximately 200 $\mu mol.m^{-2}.s^{-1}$. To ensure enough protein was obtained, cultures were grown in 2L Erlenmeyer flasks containing 1L of growth medium.

Prior to the experimental work, cultures of both 18-1T and 18-1NT were rendered axenic through antibiotic treatment (Chapter 3). Protein extraction of axenic dinoflagellate cultures will ensure that identified proteins are exclusively of dinoflagellate origin. The absence of bacteria was confirmed in each culture, prior to protein extraction, by PCR amplification of DNA extracted from 18-1 cultures as described in Martins et al (2004). Briefly, cells were collected by centrifugation and nucleic acids extracted using a 3% CTAB method (Doyle and Doyle 1990) following cell disruption in a bead beater (Biospec Products, OK). DNA was amplified with 341f and 907r prokaryotic primers, following conditions as described in (Schafer and Muyzer 2001). The absence of amplification products, as visualized in an agarose gel, confirmed that the dinoflagellate cultures were axenic.

Protein extraction

Exponentially growing cultures of 18-1T and 18-1NT were collected by centrifugation at 5000xg for 20 minutes at 4°C, in pre-chilled tubes. Cell pellets were rinsed in cold 1X PBS (Sigma Chemical Co, MO) twice before being flash frozen in liquid nitrogen and stored at -80°C, for no more than one month, until analysis. For protein extraction, pellets were re-suspended in thiourea/urea lysis buffer (2M thiourea, 7M urea, 2% CHAPS, 2% Triton X 100, 1% DTT) as described in (Gorg 2003), with the addition of protease inhibitors (TLCK 100μM, leupeptin 25 μg, aprotinin 500 U and pepstatin A 35 μg, per ml of lysis buffer). Cells were disrupted with chilled glass beads in a bead beater (Biospec Products) and lysis of the majority of cells confirmed under a light microscope. Cell debris and unbroken cells were removed by centrifugation at 40,000 x g for 1 hour at 4°C.

Proteins contained in the supernatant from the latter centrifugation step were precipitated in TCA/acetone. The protein solution was incubated at 4°C for 30 minutes in 10% TCA. Proteins were collected by centrifugation at 22,000xg for 30 minutes at 4°C. Pellets were washed 3 times in ice-cold acetone with 20mM DTT and dried in a Speed-Vac for 5 minutes with mild heat to remove residual acetone. Dried pellets were stored at -80°C until needed, for a maximum of 1 month.

DIGE

Two dimensional difference gel electrophoresis (DIGE) was performed at the W.M. Keck Facility, Yale University. The detailed methodology used can be found in the Ettan DIGE manual (Amersham Biosciences). Briefly, samples supplied as frozen pellets were resuspended in Standard Cell Lysis Buffer (Amersham Biosciences Option 2 - without thiourea). Accurate quantification was performed using amino acid analysis. 100 µg of each 18-1T and 18-1NT protein extract was labeled with Amersham Biosciences Cy-3 and Cy-5 N-hydroxysuccinimidyl ester dyes, respectively. Isoelectric focusing (IEF) was performed using pH3-10 IPG strips and the second dimension run in a 12.5% polyacrylamide gel. A fluorescent gel image was acquired by scanning at 2 wavelengths simultaneously on an Amersham Typhoon 9400 Imager and analysis was performed using the differential in-gel analysis module of the Amersham DeCyder software package. Protein spots that were identified as showing different degrees of intensity between the two samples were confirmed by visual inspection of the gel image.

Two-dimensional gel electrophoresis

2-D followed by silver staining was used to confirm the results obtained by DIGE, namely the reproducibility of the 18-1 proteome. The differentially expressed proteins identified by DIGE were further studied in several 2-D gels of 18-1T and 18-1NT cultures. To ensure comparability of all 2-D gels and to minimize variation in separated proteins due to differences in solutions, pre-made commercially available reagents were used for all gel runs. Unless stated all products and equipment were obtained from Bio-Rad (CA). Several extraction, rehydration, IEF and SDS-PAGE conditions were assayed to ensure maximum reproducibility of protein separation. The conditions described in this section were considered to be optimal, as determined after several trials.

Frozen protein pellets, obtained as described above, were re-solubilized in ReadyPrep 2-D starter kit rehydration/ sample buffer. Prior to 2-D analysis, an aliquot was removed for protein quantification, determined with a modified Bradford Assay (Bio-Rad) according to the manufacturer's specifications. Samples were quantified against a standard curve of known bovine serum albumin concentrations.

40-60 µg total protein was loaded onto 11cm IPG strips following the manufacturer's specifications. 2-D SDS-PAGE standards were added at this stage to each sample for pI calibration. The choice of pH gradient was based on preliminary observations and those of (Chan et al. 2002) that dinoflagellate proteins are mainly acidic, with pI values mostly in the 4-8 range. A pH gradient of 4-7 was ultimately chosen for all experiments.

IPG strips were rehydrated with samples for 14 - 16 hours in active conditions (50V at 21°C) in a PROTEAN IEF cell. IEF was performed at 20°C, using the following running conditions: 100V 1 hour, 250V 20 minutes linear ramp, 8000V 2.5 hours linear ramp and a final step at 8000V, 30,000V-hour rapid ramp. IPG gel strips were kept frozen overnight at -80°C immediately upon IEF completion. Gel strips were equilibrated in equilibration buffer I and II, following instructions from the manufacturer. After equilibration, strips were allowed to sit in the SDS-PAGE running buffer for a few seconds prior to blotting in filter paper to remove excess liquid. Second dimensions (SDS-PAGE) were run in a Criterion system in ready-made Bis-Tris 10% Criterion XT gels. Electrophoresis was performed at a constant voltage of 200V for a maximum of 60 minutes. Proteins were immediately fixed and visualized by silver staining (SilverQuest™ Silver Staining Kit, Invitrogen). Exposure times were kept constant between multiple gels to minimize variation due to the silver stain.

2-D was performed in duplicate for each protein sample (technical replicates) to help identify irregularities potentially occurring during IEF or SDS-PAGE that could influence results. In addition, samples were obtained from independent cultures of 18-1T and 18-1NT. Only proteins consistently identified in 3 independent biological replicates as showing different degrees of intensity between 18-1T and 18-1NT were considered to be differentially expressed.

Gels were compared to each other by eye and proteins potentially changing between the two cultures were recorded. Gel images were further analyzed using PD Quest software version 7.3.1 (Bio-Rad). Protein spots were automatically detected in cropped, normalized, low background images and different protein gels were matched to aid comparison.

Protein identification

Protein spots of interest, i.e., those differentially expressed, were further analyzed at the WM Keck Foundation, Yale University (detailed protocols found in htpp://keck.med.yale.edu/prochem/msid.htm). Identification was restricted to protein spots with a Cy5/3 absolute ratio higher than 3, corresponding to proteins with a 3-fold higher expression level in one culture. Selected proteins were automatically excised from the gel with an Ettan Spot picker (Amersham Biosciences) and trypsin-digested in a 96 well plates. Peptide mass fingerprinting (PMF) and MS/MS data were obtained on a tandem time of flight MS/MS 4700 Proteomics Analyzer (Applied Biosystems). All searches were performed in GPS Explorer (Applied Biosystems) using MASCOT analysis. Protein scores lower than 77 were considered random matches and were not regarded as positive identifications.

Due to the general lack of genomic and protein data for dinoflagellates, searches were performed against two National Center for Biotechnology information (NCBI) databases: NCBI nr first, followed by NCBI_EST_others. At the time the query was performed, the expressed sequence tag (EST) database included sequences from a few dinoflagellates

(e.g. *Amphidinium carterae* (Bachvaroff et al. 2004), *Lingulodinium polyedrum* (Tanikawa et al. 2004) and *Karenia brevis* (Lidie et al. 2005) and one member of the genus *Alexandrium* (Hackett et al. 2005). *Alexandrium tamarense* is also a saxitoxin producer. Combined PMF/ MS/MS data were used when searching the EST database.

RESULTS

DIGE

18-1T and 18-1NT protein profiles were well resolved in the DIGE gel and no difficulties were encountered during labeling of the protein extracts. Proteins were concentrated in the 4-7 pH range and the most prominent proteins migrated between 20 and 200 kDaltons.

The protein profiles obtained for 18-1T (Fig.1A) and 18-1NT (Fig.1B) were generally very similar. A total of forty protein spots showing a 3 or more-fold increase in spot volume ratio in one of the 18-1 subcultures was identified. Some of these, due to their highly similar pIs and molecular weight, as visualized in the DIGE gel, were considered to be isoforms of the same protein. This assumption was confirmed following protein identification (see below). Of the 40 proteins showing a difference in spot volume, 11 were more abundant in the toxic 18-1T *A. lusitanicum* culture, while the remainder were more abundant in the non-toxic 18-1NT strain. No protein spots were found exclusively in one of the 18-1 subcultures, though one protein spot had an expression ratio of 7.6, being higher in the toxic isolate.

Protein identification

Of the 40 protein spots in DIGE gels identified as differentially expressed in the two subcultures, a total of 12 spots were chosen for MS analysis. Two spots were picked due to their higher expression in the toxic form of 18-1 (7.6 and 6.8 fold difference, see Table 1). The remaining 7 protein spots were randomly chosen from the pool of spots showing a 3- or more -fold difference between 18-1T and 18-1NT. Additionally, three protein spots, thought to be isoforms of 3 proteins chosen above, were included for analysis.

Of the 9 unique proteins, 5 could not be identified by comparison with available databases (Table 2). This was expected due to the low number of available protein and EST sequences from dinoflagellates. Information obtained for these unknown proteins is presented in the Supplemental Information section of this Chapter. Four proteins could be identified with a high level of confidence (significant Mascot scores, $p<0.05$). Two proteins with increased expression in 18-1T were identified as chloroplast transketolase and glyceraldehyde- 3- phosphate dehydrogenase. These two proteins were similar to proteins from other species of dinoflagellates. Protein spot 1357 (and isoform 1358), also larger in 18-1T, could not be identified through comparison to the NCBInr database. However, a positive match to a *Karenia brevis* EST was found when queried against the

Table 4-1. Molecular weight, pI and expression ratio of proteins chosen for identification

Spot no.	MW/pI[a]	Cy5/Cy3 ratio[b]
198	>207/5.5	-3.0
742*	110/5.2	-7.6
1358*	50/5.2	-6.8
1583	35/6.5	-3.6
2277	<7/5.2	-3.2
622*	120/5.1	3.8
950	80/5.8	3.8
1994	20/5.5	3.9
2131	12/5.6	3.2

[a] calculated from the position in the DIGE gel. Molecular weight (MW) expressed in kD. Values are approximate

[b] 18-1T labeled with Cy3 and 18-1NT labeled with Cy5

* one putative of each of these proteins were also analyzed by MS

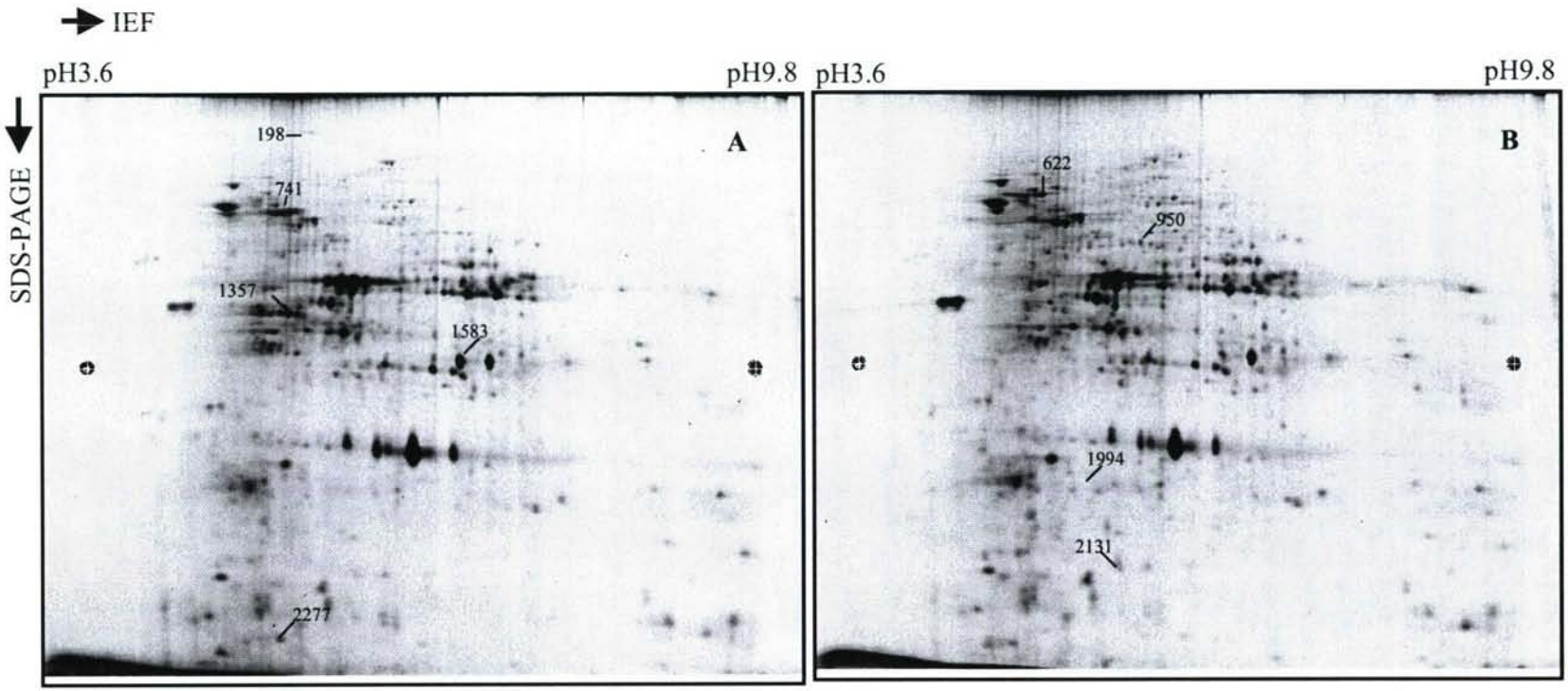

FIGURE 4-1. DIGE gels for 18-1T (A) and 18-1NT (B). Protein extracts were labeled with Cy3 (18-1T) and Cy5 (18-1NT) and gel images obtained by scanning two wavelengths. Numbers refer to the protein spots chosen for protein identification and listed on Table 1.

Table 4-2. Results of database searches for selected proteins showing 3 or more fold difference between 18-1T and 18-1NT

	NCBInr				NCBI_EST_others			
Protein spot	Protein name	Accession number	Matched peptides	Coverage %	Gene product	Accession number	Matched peptides	Coverage %
198	No match	-	-	-	No match	-	-	-
742	Chloroplast transketolase	AAW79357	10	9	cDNA *Alexandrium tamarense*	CK786508	3	18
1358	No match	-	-	-	cDNA *Karenia brevis*	CO062996	7	32
1583	Glyceraldehyde-3-phosphate dehydrogenase	AAD01872	9	29	cDNA *Alexandrium tamarense*	CK782563	7	47
2277	No match	-	-	-	No match	-	-	-
622	Heat shock protein 90	AAR27542	25	47	cDNA *Lingulodinium polyedrum*	CD810779	18	71
950	No match	-	-	-	No match	-	-	-
1994	No match	-	-	-	No match	-	-	-
2131	No match	-	-	-	No match	-	-	-

NCBI_est_other database. Later this sequence was annotated as a phosphoglycerate kinase, by searching the full *Karenia* EST against sequences in the available database.

Of the proteins shown to increase in 18-1NT, only one (and its isomer) could be identified through NCBI_nr and NCBI_est_other database searches. The identification of protein spot 623 was possible due to homology between proteins of 18-1 and proteins from other organisms. Specifically, protein spot 623 was found to be similar to heat shock protein 90 (hsp90) from *Lessardia elongata.*

As expected, protein spots thought to be isoforms had the same match in both database searches. However, the percent coverage and matched peptides varied.

2-D analysis

Protein profiles obtained through 2-D analysis of 18-1NT and 18-1T extracts were well resolved (Fig.2A and B). Visual analyses of technical and biological replicates showed a consistent profile. No major horizontal and vertical streaking was observed for any gel, showing the suitability of the extraction procedure chosen. The inclusion of a 100V step prior to IEF clearly improved background and an increase in the total voltage (30,000 to a minimum of 40,000) improved protein focusing. However, this methodology limited the amount of protein that could be loaded to 40-60 μg. Sample loading of 100 μg total protein resulted in smearing and loss of resolution. It is possible this was caused by contaminants, such as nucleic acids that could not be removed. Approximately 600 protein spots were found by PDQuest in most gels.

The protein profiles were highly reproducible with respect to the major proteins. Some variability occurred with protein spots at low and high molecular weight and at the extremes of the pH gradient. Comparison of 2-D gels obtained from 18-1T and 18-1T revealed that most proteins are highly shared between the two subcultures, agreeing with the results from DIGE analysis.

Protein spots identified as being differentially expressed in DIGE analysis were specifically searched for in 2-D gels. Calibration of pI and molecular weight using 2-D standards and the similar protein profiles obtained using DIGE and 2-D gels allowed the comparison of protein profiles obtained with each method. However, it was not possible to indisputably identify the DIGE protein spots 198, 2277 and 1994 in various 2-D gels. This was mainly due to the lower reproducibility of runs at the extremes of the molecular weight range as well as the lack of recognizable protein patterns in these sections of the 2-D gels.

Protein spots identified from the DIGE analysis as heat shock proteins were highly expressed in the non-toxic 18-1NT in all 2-D gels analyzed (Fig.3A). This is a stable characteristic of 18-1NT although the culture was maintained in optimal growth conditions. Protein spots 950 and 2131, identified in DIGE as more abundant in 18-1NT were confirmed by 2-D gel analysis (Fig.3B, E, respectively). Proteins identified as glyceraldehyde-3-phosphate dehydrogenase (Fig. 3D), chloroplast transketolase (Fig. 3A)

and phosphoglycerate kinase (Fig. 3C) were consistently found to be more abundant in the toxic 18-1T, as observed in multiple 2-D gels. For the proteins above, 2-D allowed the confirmation of DIGE results in several biological replicates.

DISCUSSION

The study of genes involved in the saxitoxin biosynthetic pathway has partially been hindered by the constitutive nature of toxin production in dinoflagellates. Furthermore, it has not been technically feasible to create and identify toxin-deficient mutant cells from this class of algae. However, in a recent study (Martins et al, 2004) a culture of *A. lusitanicum* that has lost the ability to produce toxins was identified. This culture is further characterized by a reduction in growth capability; the relation of this to loss of toxicity is unknown. A subculture of this isolate maintains the ability to produce toxins and is characterized by both a higher growth rate and a higher maximum cell density.

This pair of cultures is an ideal experimental system in which to study the genes involved in toxin production and growth. A previous study demonstrated that the observed differences in phenotype are most likely a property of the dinoflagellate itself, rather than a result of differences in the bacterial assemblage (Chapter 3). Based on these observations, protein expression was analyzed in the two 18-1 cultures grown under the same conditions, but where both toxicity and growth vary. In particular, the proteomes of 18-1T and 18-1NT should reflect both transcriptional and post-transcriptional regulation of genes that ultimately are the basis of the loss of toxicity and the reduction in growth capability. In this study, the proteins expressed by each 18-1 subculture were reproducibly separated by both 2-D and DIGE. The combination of these two techniques allowed the identification of differentially regulated proteins between two subcultures of the same *A. lusitanicum* isolate that differ in the ability to produce toxins and growth.

Methodological considerations

Both DIGE and 2-D allowed the resolution of proteins from 10 to 210 kDa, indicating no protein losses at specific molecular weights or pI. 2-D analysis data for investigation of protein expression in dinoflagellates (Chan et al. 2002) is scarce but provides a basis for the development of methodology. As expected, a TCA/acetone protein extraction method worked well for algal extracts. However, sample preparation previously described for other species of *Alexandrium* (Chan et al. 2005a) could not be directly applied to 18-1 samples and different extraction buffers had to be evaluated. This highlights the need to optimize protein extraction from case to case.

As a result of these changes in methodology, though the protein loading range and protein profile resolution was comparable to those previously observed for *Alexandrium* (Chan et al. 2005a), more protein spots were reported in the current study. This could be explained by the use of thiourea in combination with urea and other detergents in the lysis buffer, which should solubilize more hydrophobic proteins.

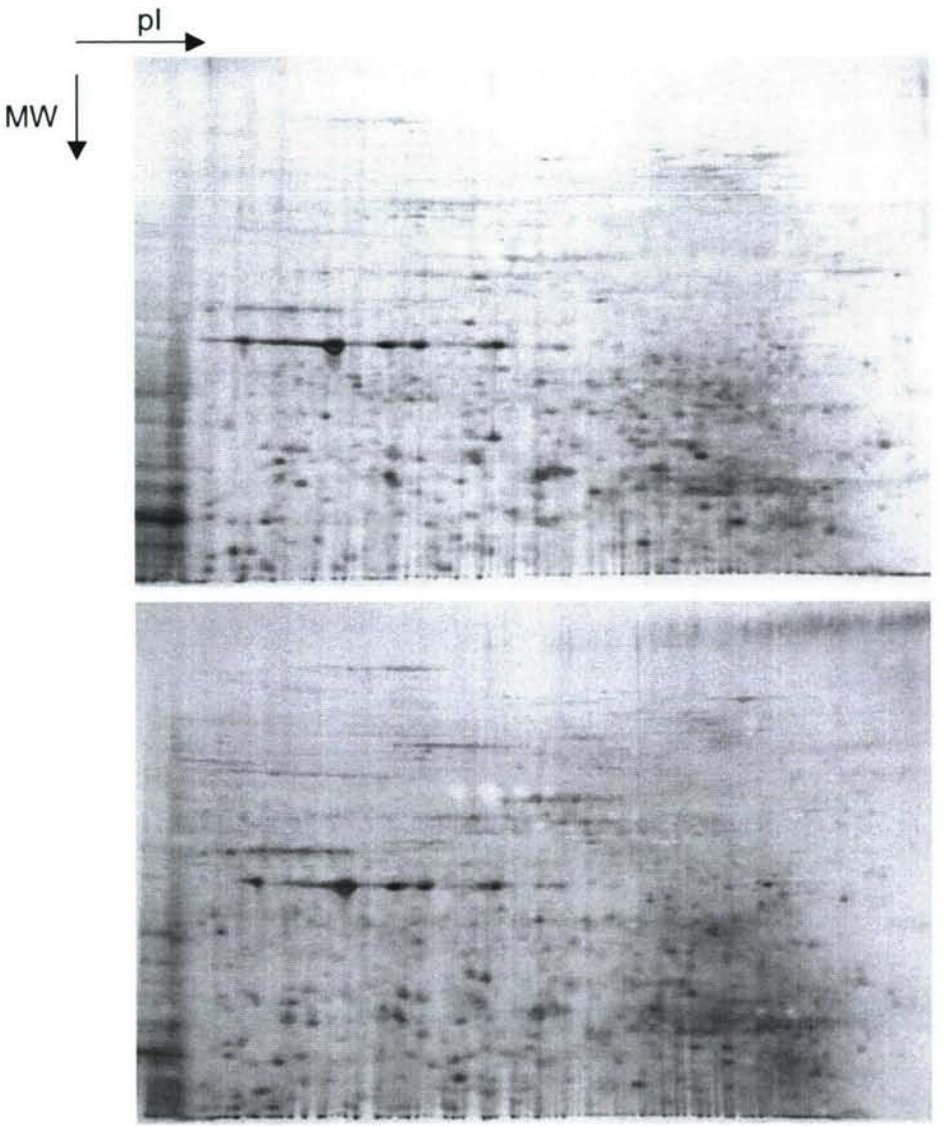

FIGURE 4-2. Representative 2 dimension gel electrophoresis images obtained for protein extracts of the dinoflagellate 18-1T (top) and 18-1NT (bottom). First dimension was run in pH3-10 IPG strips and second dimension on a 12.5% polyacrylamide gel. Protein spots are visible by silver staining. (MW: molecular weight)

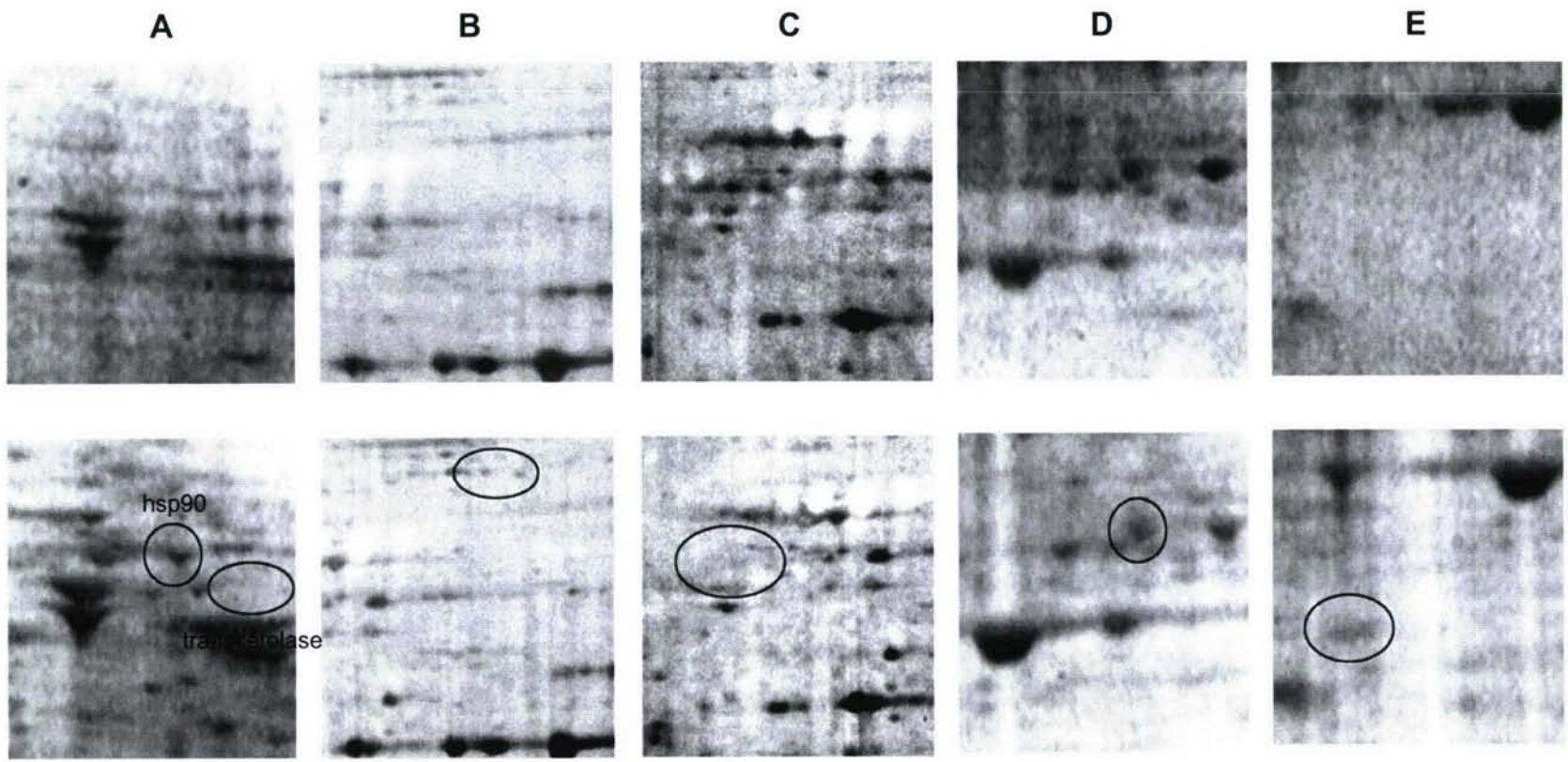

FIGURE 4-3. Detail of 2-D gels obtained from 18-1T and 18-1NT showing proteins of interest from DIGE analysis. IEF was performed over a pH range of 4-7 and second dimension ran in a 10% SDS-PAGE. Gels were silver stained. Details are representative of a least 2 gels. (A) hsp90 and chloroplast transketolase expression in 18-1T, top and 18-1NT, bottom; (B) protein 950 expression in 18-1T, top and 18-1NT, bottom; (C) phosphoglycerate kinase expression in 18-1T, top and 18-1NT, bottom; (D) GAPDH expression in 18-1T, top and 18-1NT, bottom; (E) protein 2131 expression in 18-1T, top and 18-1NT, bottom.

The proteins identified to be of interest through DIGE analysis were of relatively high abundance and therefore the limited total protein loading range of 40-60 μg onto each gel did not constitute a problem. However it is acknowledged that the entire range of proteins expressed by 18-1NT and 18-1T could not be detected in this single study. Proteins of low abundance were arguably not detected using the current methodology, if we accept that differences in protein abundance in one organism can range from 6 to 8 fold (Peck 2005). If low abundance proteins are to be analyzed, shorter pI range IPG strips could allow for more protein to be loaded following our extraction procedure.

Regardless of these caveats, our methodological approach allowed the identification of several proteins that were differentially expressed between the toxic and non-toxic variants of 18-1.

Protein expression analysis

Like other eukaryotes, dinoflagellates have been demonstrated to employ both transcriptional and translational regulation. (Erdner and Anderson 2006) have recently shown dinoflagellates to exhibit transcriptional complexity similar to that of multicellular eukaryotes. Some genes are specifically known to be regulated at the transcriptional level, including S-adenosylhomocysteine hydrolase, methionine aminopeptidase and a histone-like protein (Taroncher-Oldenburg and Anderson 2000). However, studies of dinoflagellate gene expression also revealed that post-transcriptional regulation occurs (Fagan et al. 1999). In view of this, analysis of differential gene expression in 18-1T and 18-1NT solely at the transcriptional level would potentially miss genes that did not employ this type of regulation. A comprehensive analysis of the proteome is therefore a necessary approach when analyzing potential differences between 18-1T and 18-1NT.

The study of the proteome has a further advantage if a point mutation is responsible for the loss of saxitoxin production. A point mutation might not result in altered mRNA transcription, but there is a higher chance that different proteins will be produced. In the case of aflatoxin- vs non-aflatoxin -producing species of *Aspergillus*, for example, the inability to produce toxins seems to be related to a single nucleotide change resulting in the loss of 65 amino acids of one expressed protein (Chang 2004). Nevertheless, proteomic analysis could still miss a non-functional enzyme if, for example, a change in one amino acid in the active site rendered the enzyme inactive but did not involve a major change in the overall pI and/or MW of the protein.

The proteomic analysis of *Alexandrium* 18-1 subcultures successfully identified six proteins that are differentially expressed in the two cultures. These proteins were consistently detected in both biological and technical replicates of each 18-1 culture, demonstrating they are truly differentially expressed. The success of this approach supports the use of 2-D analysis for study many aspects of dinoflagellate biology. One example is the identification of proteins involved in response to nutrient stress.

Preliminary results allowed visualization of protein spots in 2-D gels which were consistently less abundant in phosphate limited cultures (Fig.4).

Protein identification

Dinoflagellates are poorly characterized organisms in genetic terms. Full genome information is not scheduled or anticipated to be available in the near future, most likely due to the unique characteristics of these organisms, which include enormous DNA content (Rizzo 1987) and widespread gene duplication (Erdner and Anderson 2006; Le et al. 1997). In addition, full sequences for specific genes are not widely available, as these seem only to be obtained on a case by case basis, when certain genes of interest are studied. Not only gene but also protein sequences are missing from the database. Dinoflagellate proteomics is a very new field and consequently a very small number of expressed proteins are described (Chan et al. 2002). In fact, the current database does not contain more than a few hundred proteins described from dinoflagellates, the majority of which are related to photosynthetic functions and bioluminescence and most being the same protein identified from different species. This general lack of information is an obstacle for most studies relying on gene discovery. For proteomic research and protein identification, gene information is particularly crucial.

Recently, however, EST libraries have become available for a few dinoflagellate species. Two major EST sequencing projects have been completed – one for *Karenia brevis* (Lidie et al. 2005) and one for *Alexandrium tamarense*. The more than 6,723 ESTs now available for the latter (Hackett et al. 2005) are particularly valuable to this study as the strain of *A. tamarense* that was sequenced is also a saxitoxin producer. It was hypothesized that the EST libraries now available for dinoflagellates would allow the identification of proteins using PMF and MS/MS data. Such an approach has been previously used for organisms other than dinoflagellates with a reasonable level of success (Kwon et al. 2003).

The feasibility of using ESTs to identify proteins from dinoflagellates was first investigated by re-analyzing existing data in the literature in light of our hypothesis. A study by (Chan et al. 2005b) showed that proteins isolated from 2-D gels can not be identified by searches against the Protein DataBank and Swiss-Prot by BLAST. However, tBLASTn searches of these same proteins performed in this thesis revealed that they match ESTs from *A. tamarense* (3 mismatches in 30 amino acids), *Lingulodinium polyedrum* (9 mismatches in 30 amino acids) and *Amphidinium carterae* (12 mismatches in 30 aa). This was an encouraging result for our purposes as it indicated that searches against ESTs can be used to identify proteins isolated from dinoflagellates.

Secondly, there was no information available on how similar *A. lusitanicum* and other dinoflagellates were in terms of expressed genes. In other words how useful would the ESTs from a related organism be in identifying proteins from the experimental system? To investigate this, a small cDNA library of *A. lusitanicum* (191 sequences, average sequence length of 260bp) (C. Martins, unpublished data) was constructed. tBlast searches revealed 50% of the sequences matched ESTs from dinoflagellates and other

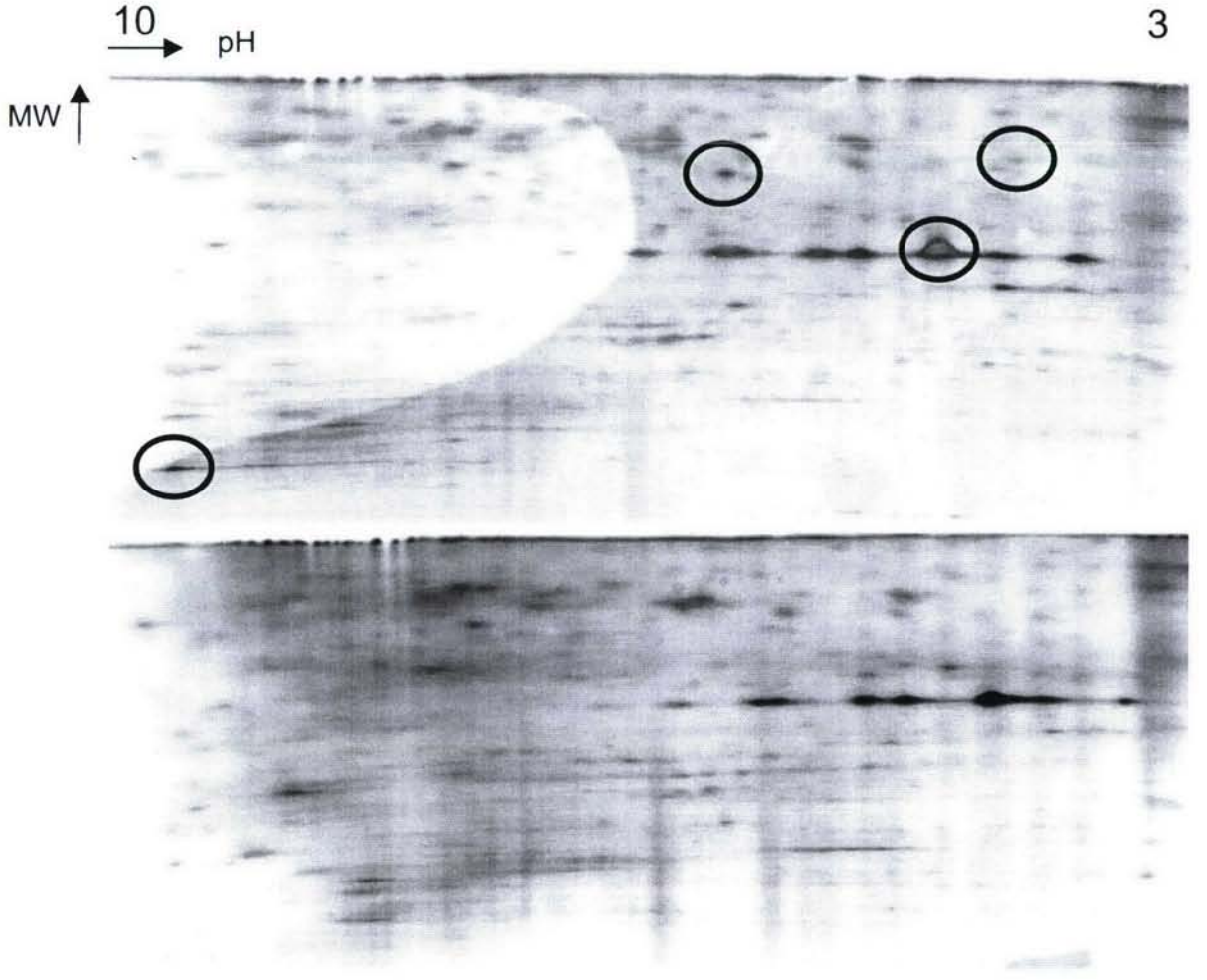

FIGURE 4-4. Representative 2-D gels obtained for A. lusitanicum 18-1T grown in nutrient replete (above) and phosphate deplete conditions (below). First dimension was performed in pH3-10 IPG strips and the second dimension in a 12.5% polyacrylamide gel. Protein spots circled have been found to be consistently down regulated under phosphate limitation in a minimum of two biological replicates.

organisms (cut-off 10^{-4} for similarity, minimum sequence length of 50bp, excluding polyA tail).

As predicted, certain protein spots of interest from our DIGE gels could be reliably identified using ESTs not only from dinoflagellates but also from other organisms. This has shown the value and utility of the growing number of ESTs in reliably identifying proteins from dinoflagellates, even though full genome information will not be available for some time.

A final comment relates to the inability to identify all proteins of interest from our proteomic analysis. Undoubtedly this is due to the still limited number of ESTs available. The current database of dinoflagellate expressed genes implies that species or strain-specific proteins, especially those involved in physiological and biochemical mechanisms restricted to dinoflagellates, are unlikely to be identified. Saxitoxin production, for example, will be such a case, as only one saxitoxin producing *Alexandrium* can so far be found in the database. More genetic information is obviously needed for the proteomic study of saxitoxin-producing organisms.

Biological role of proteins of interest

It was possible to identify some of the proteins differentially expressed in the toxic and non-toxic 18-1 subcultures. These identifications suggest the potential biological function of the proteins and consequently may help to explain why they are differentially expressed in the experimental system.

Due to its potential use in phylogenetic studies, heat shock protein 90 (hsp90) has been characterized for many dinoflagellates (Leander and Keeling 2004). Heat shock proteins are a group of proteins that aid in protein folding. In addition, under normal conditions, hsp90 acts to buffer hidden genetic variability in organisms (Ruden et al. 2005). Though common in cells under optimal conditions, higher expression of hsp90 has been linked to stress responses in many organisms. It is unclear why hsp90 is consistently highly expressed in the non-toxic, slower-growing 18-1NT, particularly as this culture was maintained in normal growth conditions, with no known stressors. Nevertheless, the slow growth indicates that some aspect of cellular metabolism was hindered. It is possible the higher expression of hsp90 in 18-1NT is in response to a protein misfolding occurring in the cells as a results of a mutation.

Proteins identified as being highly expressed in the toxic strain compared to the non-toxic are related to cellular metabolism. In particular, all 3 enzymes identified (glyceraldehyde 3-phosphate dehydrogenase (GAPDH), transketolase and phosphoglycerate kinase) are part of the Calvin cycle, used by algae, cyanobacteria and higher plants to convert carbon dioxide into carbohydrates. The observation that 3 enzymes, all involved in the same cycle, are down-regulated in the slower growing non-toxic 18-1 strongly suggests that the Calvin cycle is functioning non-optimally in these cells. A stronger evidence for this suggestion is the fact that transketolase is one of the enzymes described as having significant control over carbon fixation (Raines 2003). Other limiting enzymes of the

Calvin cycle have not been identified. However, it should be noted that only 12 of the 40 proteins recognized in the DIGE analysis as differentially expressed in the two subcultures were further identified by MS analysis. Therefore, it is feasible that other enzymes involved in the Calvin cycle are amongst the proteins not submitted for identification. This can only be resolved by a more comprehensive analysis of all differentially expressed proteins.

If indeed the Calvin cycle is down regulated in 18-1NT cells, the most parsimonious explanation is that this is co-related with other observations made for the 18-1NT subculture, in terms of its ability to photosynthesize. Preliminary data has shown that 18-1NT has a lower chlorophyll content per cell that the toxic counterpart (C. Gameiro, unpublished data). This is not totally unexpected, as 18-1NT was subjected to antibiotic treatment for a long period of time. It has previously been shown that antibiotics can cause bleaching of algal cells, by acting directly on the chloroplasts (Miyoshi and Tsubo 1969), for example by inhibiting chlorophyll formation or influencing protein synthesis in the organelle. These effects have been shown to continue even after the antibiotic treatment has been discontinued (Provasoli et al. 1948). It can be hypothesized that the photosynthetic apparatus of 18-1NT has been affected by the years of antibiotic treatment which could have either reduced the number of chloroplasts or reduce photosynthetic efficiency. The Calvin cycle would appear reduced as a consequence in both of these cases in cells of 18-1NT in comparison with 18-1T.

In summary, there are several potential explanations for the observation that enzymes of the Calvin cycle are less expressed in cells of 18-1NT compared to 18-1T. On one hand, the Calvin cycle could be directly down regulated in the non-toxic subculture, for example through one of the enzymes of the cycle. Alternatively, the reduction in the Calvin cycle could be a consequence of the lower efficiency or of the lower number of chloroplasts in 18-1NT cells, as discussed above. If these explanations are to be differentiated, further proteomic work on the two subcultures of 18-1 could entail fractionation of the total cellular protein and the study of the chloroplast fraction in isolation.

The lower efficiency of the Calvin cycle also could provide a potential explanation for the growth patterns observed for the non-toxic culture. In fact, 18-1NT is characterized not only by a slower growth rate but also by a lower maximum cell density than its toxic counterpart. The former characteristic is indicative that 18-1NT growth is limited by the absence or inability to fully utilize a certain nutrient. Both subcultures were grown in nutrient-rich seawater medium with adequate light conditions, yet the same conditions did not limit 18-1T. Having observed that the Calvin cycle appears to be reduced, it is hypothesized that 18-1NT growth is being limited by the inability of the cells to efficiently use CO_2. In other words, the growth patterns observed in 18-1NT could be explained by the results obtained by the proteomic analysis performed. It should also be noted that these enzymes are also part of glycolysis (GAPDH and phosphoglycerate kinase) and the pentose phosphate pathway (transketolase), both of which occur in all cells.

CONCLUSIONS

This study has demonstrated the suitability of proteomic analysis, specifically of DIGE, to the study of proteins differentially regulated in dinoflagellates. The comparison of PMF and MS/MS data obtained for proteins of interest allowed the identification of some of these proteins, observed to be down-regulated in 18-1NT cultures. These were homologous to enzymes involved in the Calvin cycle and therefore can potentially explain the reduced growth capability observed in the 18-1NT isolate.

However, identification of proteins directly related to the saxitoxin biosynthetic pathway was not achieved in this study. Previous studies have attempted to use proteomic techniques to detect biomarkers of toxicity in dinoflagellates of the species *A. tamarense* and *A. minutum* (Chan et al. 2005b). In that study, 3 protein spots were identified in 6 toxic strains of these dinoflagellates but not in one non-toxic *A. tamarense* tested. The present study did not detect these proteins as being differentially expressed. Proteins of similar molecular weight and pI were present in both 18-1T and 18-NT proteome. Furthermore, tblastn searches of these proteins revealed they match ESTs from *A. fundyense* (3 mismatches in 30 aminoacids), *Lingulodinium polyedrum* (9 mismatches in 30 aminoacids) and *Amphidinium carterae* (12 mismatches in 30 aa). Since the two former genera of dinoflagellates have never been linked to production of saxitoxin, it is unlikely these proteins are directly linked to that biosynthetic pathway.

Dinoflagellate proteomic analysis is a powerful technique. However, the benefits from protein identification in dinoflagellates are not limited to the possible discovery of proteins involved in growth or the toxin synthesis pathway. The identification of expressed proteins can be connected to genes and provide information on an organism whose full genome sequence is many years away.

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REFERENCES

Anderson, D. M. 1991. Toxin variability in *Alexandrium* : Mechanisms and implications, p. 9. *In* J. M. Fremy [ed.], Proceedings of a Symposium on Marine Biotoxins, Paris, 30-31 January 1991.

Bachvaroff, T. R., G. Concepcion, C. R. Rogers, E. M. Herman, and C. F. Delwiche. 2004. Dinoflagellate expressed sequence tag data indicate massive transfer of chloroplast genes to the nuclear genome. Protist **155:** 65-78.

Cembella, A. D. 1988. Toxicity of cultured isolates and natural populations of *Protogonyaulax tamarensis* (Lebour) Taylor from the St. Lawrence Estuary. Journal of Shellfish Research **7:** 611-622.

Chan, L. L. and others 2005a. Use of two-dimensional gel electrophoresis to differentiate morphospecies of *Alexandrium minutum*, a paralytic shellfish poisoning toxin-producing dinoflagellate of harmful algal blooms. Proteomics **5:** 1580-1593.

Chan, L. L., I. J. Hodgkiss, S. Lu, and S. C. Lo. 2004. Use of two-dimensional gel electrophoresis proteome reference maps of dinoflagellates for species recognition of causative agents of harmful algal blooms. Proteomics **4:** 180-192.

Chan, L. L., S. C. Lo, and I. J. Hodgkiss. 2002. Proteomic study of a model causative agent of harmful red tide, *Prorocentrum triestinum* I: Optimization of sample preparation methodologies for analyzing with two-dimensional electrophoresis. Proteomics **2:** 1169-1186.

Chan, L. L. and others 2005b. Identification and characterization of a "biomarker of toxicity" from the proteome of the paralytic shellfish toxin-producing dinoflagellate *Alexandrium tamarense* (Dinophyceae). Proteomics **6:** 654-666.

Chang, P. 2004. Lack of interaction between AFLR and AFLJ contributes to nonaflatoxigenicity of *Aspergillus sojae*. Journal of Biotechnology **107:** 245-253.

Doyle, J. J., and J. L. Doyle. 1990. Isolation of plant DNA from fresh tissue. Focus **12:** 13-15.

Erdner, D., and D. M. Anderson. 2006. Global transcriptional profiling of the toxic dinoflagellate *Alexandrium fundyense* using Massively Parallel Signature sequencing. BMC Genomics **7:** 88.

Fagan, T., D. Morse, and J. W. Hastings. 1999. Circadian Synthesis of a Nuclear-Encoded Chloroplast Glyceraldehyde-3-Phosphate Dehydrogenase in the Dinoflagellate *Gonyaulax polyedra* is Translationally Controlled. Biochemistry (Washington) **38:** 7689-7695.

Gorg, A. 2003. Two-Dimensional Electrophoresis with Immobilized pH Gradients for Proteome Analysis, p. 77.

Graves, P., and T. Haystead. 2002. Molecular Biologist's Guide to Proteomics. Microbiology and Molecular Biology Reviews **66:** 39-63.

Guillard, R. R. L. 1975. Culture of phytoplankton for feeding marine invertebrates, p. 29-60. *In* W. L. Smith and M. H. Chanley [eds.], Culture of marine invertebrate animals. Plenum Publishing Corporation.

Hackett, J. and others 2005. Insights into a dinoflagellate genome through expressed sequence tag analysis. BMC Genomics **6:** 80.

Kellman, and Nielan. 2006 (oral presentation). 12th International Conference on Harmful Algae.

Kwon, K. H., M. Kim, J. Y. Kim, S. Kim, Y. M. Park, and J. S. Yoo. 2003. Efficiency improvement of peptide identification for an organism without complete genome sequence, using expressed sequence tag database and tandem mass spectral data. Proteomics **3:** 2305-2309.

Le, Q. H., P. Markovic, J. W. Hastings, R. V. M. Jovine, and D. Morse. 1997. Structure and organization of the peridinin-chlorophyll a-binding protein gene in *Gonyaulax polyedra*. Molecular and General Genetics **255:** 595-604.

Leander, B. S., and P. J. Keeling. 2004. Early evolutionary history of dinoflagellates and apicomplexans (Alveolata) as inferred from hsp90 and actin phylogenies. Journal of Phycology **40:** 341-350.

Lee, D.-H., M. Mittag, S. Sczekan, D. Morse, and J. W. Hastings. 1993. Molecular cloning and genomic organization of a gene for luciferin-binding protein from the dinoflagellate *Gonyaulax polyedra*. Journal of Biological Chemistry **268:** 8842-8850.

Lidie, K. B., J. C. Ryan, M. Barbier, and F. Van Dolah. 2005. Gene expression in Florida red tide dinoflagellate *Karenia brevis*: analysis of an expressed sequence tag library and development of DNA microarray. Marine Biotechnology **7:** 481-493.

Martins, C. A., D. Kulis, S. Franca, and D. M. Anderson. 2004. The loss of PSP toxin production in a formerly toxic *Alexandrium lusitanicum* clone. Toxicon **43:** 195-205.

Mascarenhas, V., P. Alvito, S. Franca, I. Sousa, J. A. Rodriguez-Vasquez, and A. G. Martinez. 1995. The dinoflagellate *Alexandrium lusitanicum* isolated from the coast of Portugal: observations on toxicity and ultrastructure during growth phases, p. 71-76. *In* P. Lassus, G. Arzul, E. Erard-Le Denn, P. Gentien and C. Marcaillou-Le Baut [eds.], Harmful Marine Algal Blooms/Proliferation d'Algues Marines Nuisibles. Proceedings of the Sixth International Conference on Toxic Marine Phytoplankton, Nantes (France), Oct 1993. Lavoisier.

Miyoshi, Y., and Y. Tsubo. 1969. Permanent bleaching of *Euglena* by chloramphenicol. Plant & Cell Physiology **10:** 221-225.

Peck, S. C. 2005. Update on Proteomics in *Arabidopsis.* Where do we go from here? Plant Physiology **138:** 591-599.

Plumley, F. G. 1997. Marine algal toxins: biochemistry, genetics, and molecular biology. Limnology and Oceanography **42:** 1240-1251.

---. 2001. Purification of an enzyme involved in saxitoxin synthesis. Journal of Phycology **37:** 926-931.

Provasoli, L. 1963. Growing marine seaweeds, p. 9-17, 4th International Seaweed Symposium.

Provasoli, L., S. Hutner, and A. Schatz. 1948. Streptomycin-induced chlorophyll-less races of *Euglena.* Proc. Soc. Exp. Biol. and Med. **69:** 279-282.

Raines, C. 2003. The Calvin cycle revisited. Photosynthesis Research **75:** 1-10.

Rizzo, P. 1987. Biochemistry of the dinoflagellate nucleus, p. 143-173. *In* F. Taylor [ed.], The biology of dinoflagellates. Blackwell Scientific.

Ruden, D. M., L. Xiao, M. D. Garfinkel, and X. Lu. 2005. Hsp90 and environmental impacts on epigenic states: a model for the trans-generational effects of DES on uterine development and cancer. Human Molecular Genetics **14:** 149-155.

Schafer, H., and G. Muyzer. 2001. Denaturing gradient gel electrophoresis in marine microbial ecology, p. 425-468. *In* J. Paul [ed.], Methods in Microbiology. Academic Press.

Scholin, C. A., M. Herzog, M. Sogin, and D. M. Anderson. 1994. Identification of group- and strain-specific genetic markers for globally distributed *Alexandrium* (Dinophyceae). 2. Sequence analysis of a fragment of the LSU rRNA gene. Journal of Phycology **30:** 999-1011.

Shimizu, Y., M. Norte, A. Hori, G. Almoursi, and M. Kobayashi. 1984. Biosynthesis of saxitoxin analogues: the unexpected pathway. Journal of the American Chemical Society **106:** 6433-6434.

Tanikawa, N., H. Akimoto, K. Ogoh, W. Chun, and Y. Ohmiya. 2004. Expressed sequence tag analysis of the dinoflagellate *Lingulodinium polyedrum* during dark phase. Photochemistry and Photobiology **80:** 31-35.

Taroncher-Oldenburg, G., and D. M. Anderson. 2000. Identification and characterization of three differentially expressed genes, encoding S-adenosylhomocysteine hydrolase, methionine aminopeptidase, and a histone-like protein, in the toxic dinoflagellate *Alexandrium fundyense*. Applied and Environmental Microbiology **66:** 2105-2112.

Taylor, F. J. R., and Y. Fukuyo. 1998. The neurotoxigenic dinoflagellate genus *Alexandrium* Halim: General Introduction. *In* D. M. Anderson, A. Cembella and G. Hallegraeff [eds.], Physiological Ecology of Harmful Algal Blooms. NATO ASI Series. Springer-Verlag.

Supplemental information

Protein spot	Observed m/z
950	940.55; 996.59; 2286.10; 982.57; 1051.70; 1165.74; 1279.76; 1448.91; 1486.76; 1649.79; 1760.91; 2448.65
1994	996.58; 1051.69; 1165.72; 1180.52; 1182.67; 1214.63; 1251.68; 1279.76; 1417.75; 1448.90; 1559.82; 1562.92; 1660.87; 1674.88; 1679.78; 1809.88; 2206.12
2131	996.59; 1031.52; 1044.02; 1045.57; 1051.70; 1165.73; 1227.76; 1229.78; 1279.76; 1391.79; 1448.91; 1561.93

CHAPTER 5

IDENTIFICATION OF METABOLITES FROM A NON-TOXIC *ALEXANDRIUM LUSITANICUM* POTENTIALLY RELATED TO SAXITOXINS

ABSTRACT

Paralytic shellfish poisoning (PSP) toxins are a family of more than 20 neurotoxins produced by dinoflagellates and a few species of freshwater cyanobacteria. The suite of toxin congeners produced by an alga can be identified through the use of analytical techniques such as high performance liquid chromatography (HPLC), which detect toxins in the form of fluorescent derivatives.

Toxin profiles and the ability to produce toxins are considered a constitutive characteristic of dinoflagellates. However, a culture of *Alexandrium* has been demonstrated to have lost the ability to produce toxins, completely lacking the gonyautoxins that were characteristic of this strain. HPLC analysis revealed further departures in the biochemical composition of this culture as compounds eluting close to neosaxitoxin and saxitoxin standards were observed. These compounds are unique to this non-toxic strain, relative to the toxin strain, and their emergence may be correlated to a disruption of the biosynthetic pathway for PSP toxins.

Studies were performed to characterize the unknown compounds. Results demonstrate that these are not saxitoxin, as the peaks are unstable in hydrochloric acid. Furthermore, retention times of the unknown compounds in high performance liquid chromatography analysis do not correspond to any of the known PSP toxins. Biological assays confirmed the lack of toxicity and sodium channel blocking activity in cell extracts. These results taken together indicate the unknown compounds are not any of the previously described PSP toxins. Additional tests suggest that the compounds share some characteristics and potentially structural similarities with PSP toxins. Like these toxins, the unknown compounds lack natural fluorescence and increase in the cell under phosphate limited conditions.

INTRODUCTION

Paralytic shellfish poisoning (PSP) toxins are a family of potent neurotoxins produced by dinoflagellates (Cembella, 1998) and a few species of freshwater cyanobacteria (Carmichael 1994). These compounds are heterocyclic guanidines that bind to sodium channels responsible for the flux of sodium in and out of nerve and muscle cells. More than 20 saxitoxin derivatives have been identified (Cembella 1998) since the isolation of the first in 1984 (Schantz 1984). These toxins are all structurally related but have different physical, chemical and pharmacological characteristics (Shimizu 2001). Previously undescribed structures continue to be reported from dinoflagellates (Negri et al. 2003) and cyanobacteria (Onodera et al. 1997), adding to the suite of PSP congeners produced by algae.

Depending on the amount and combination of toxins produced, an alga can be characterized with respect to its toxin composition or profile. Toxin profiles can be obtained through the use of analytical techniques, optimized to detect PSP toxins and its derivatives. High performance liquid chromatography (HPLC) can detect PSP toxin congeners in the form of fluorescent derivatives. A widely used post-column oxidation system uses 3 different mobile phases and has high sensitivity for the detection of PSP toxins (Oshima 1995). The application of this method requires three chromatographic runs using three different mobile phases to separate the known toxins: basic saxitoxins (neosaxitoxin, decarbamoylsaxitoxin and saxitoxin) in one; the gonyautoxins (GTX1-5) in another and C-toxins (C1-C4) in another. Each compound will have a specific retention time and identification is possible through comparison to standards of toxin congeners. In addition, a variety of easily performed modifications to either the sample preparation or HPLC mobile phases can be carried out such that PSP toxins are more conclusively identified.

HPLC has been widely used to characterize the toxin profile of cultures of dinoflagellates. Analysis of several isolates revealed that under normal growth conditions, profiles could be considered characteristic of a culture (Cembella et al. 1987). Slight variations do occur, typically following extended nutrient limitation (Anderson et al. 1990a). Recently, an *Alexandrium lusitanicum* culture, originally isolated in 1962, was found to be an exception to this rule. The toxin content and toxin profile of this culture have been measured and documented by different authors (Cembella et al. 1987; Mascarenhas et al. 1995). These studies indicated that the toxin profile of *A. lusitanicum* 18-1 was highly stable over the years, comprising only of 11-hydroxysulfate PSP toxins, namely gonyautoxin 1 to 4 (GTX1-4). This was the case until 2000, when routine testing for toxicity using HPLC found no evidence of PSP toxins. However, a subculture of this same strain, which had been maintained in an US laboratory, maintained the same toxin profile and roughly the same toxin content as the parental culture. These subcultures have been designated 18-1NT and 18-1T, respectively, to reflect the differences in toxicity (Martins et al. 2004).

Although 18-1NT completely lacked the GTX that were characteristic of this strain, it did contain fluorescent compounds that were separated with the HPLC mobile phase for basic saxitoxins. The presence of these peaks is a departure from the biochemical composition previously observed for the 18-1 strain. In fact, these compounds are unique to the non-toxic strain and are not observed in the toxic variant of 18-1, despite the two strains being subcultures of the same isolate. Furthermore these peaks were not found in any other *Alexandrium* extracts analyzed (D. Kulis, unpublished data). A similar chromatographic profile was obtained for all cell extracts of 18-1NT, indicating that the presence and the elution time of the unknown compounds are a stable characteristic of the non-toxic isolate. Further studies were performed to determine whether the compounds were any of the known PSP toxins and attempts were made to determine their identity. The possible link of these compounds to the loss of toxin production ability is discussed.

MATERIAL AND METHODS

Dinoflagellate cultures

The parental *A. lusitanicum* 18-1 culture was originally isolated from Lagoa de Obidos, Portugal, in 1962 and shown to be toxic by mouse bioassay. At some unknown point between 1995 (Franca et al. 1995); and 2000 (Pereira et al. 2000), the 18-1 culture became non-toxic. For discussion purposes, this culture is now referred to as 18-1NT to distinguish it from the parent culture. A subculture of the original isolate also exists that maintains the same toxin profile and roughly the same toxin contents as the original 18-1, and it was renamed 18-1T (Martins et al. 2004).

For all experiments in this study, cultures of the non-toxic variant, 18-1NT, were maintained and grown in f/2 growth medium (Guillard and Ryther 1962) at 20 ± 1°C on a 16:8 L:D cycle. All cultures were maintained in flasks containing 1L of growth medium, made up in filtered Vineyard Sound, MA, seawater and autoclaved before use.

Low phosphate experiment

To study the effect of phosphate stress on the per cell concentration of the unknown compounds, cultures of 18-1T and 18-1NT were grown in local sterile seawater enriched with normal f/2 media components, excluding phosphate. Phosphate was then added at 1/40 the concentration of the full strength f growth medium (1.8 uM PO_4^{-3}). The phosphorous-limited cultures were considered replete for nitrate.

Cultures for each condition (n=2) were started with nutrient replete inocula of exponentially growing 18-1, at a starting density of around 1000 cells/ml. Growth was monitored through periodic cell counts in a Sedgwick-Rafter counting chamber. Once during each of the lag (i.e., day 5), exponential (day 16) and stationary phases (day 39), 15 ml of culture were removed aseptically for analysis by HPLC. Control cultures were maintained in f/2 sterile medium.

HPLC analysis

Prior to HPLC analysis, two 1 ml aliquots were removed from mixed cultures and preserved in 20 µl Utermöhl's solution (Utermöhl 1958) for cell counts in a Sedgwick-Rafter counting chamber. At least 400 cells were counted in each chamber and the culture's cell concentration was determined as the average of the two cell counts. Variations in duplicate cell counts were generally less than 20%.

Fifteen ml of cultures were harvested by centrifugation at 5,000g for 5 minutes at room temperature and cell pellets were ressuspended in 1ml of 0.5M acetic acid. Cells were disrupted by sonication (10W, 20s) on a frozen block and the resulting crude extract was stored at -20°C prior to analysis. HPLC analysis was performed according to the method of (Oshima 1995), as described in (Anderson et al. 1994) to detect and quantify basic toxins: saxitoxin (STX), neosaxitoxin (neoSTX) and decarbamoylsaxitoxin (dcSTX). Toxins were separated using a mobile phase of 2mM sodium heptane sulfonate in 30mM ammonium phosphate, pH 7.1 with 5% acetonitrile, as described by Oshima (1995). Mixtures of PSP toxin standards were kindly provided by Prof. Oshima (Tohoku University, Sendai, Japan).

RESULTS AND DISCUSSION

The 18-1NT culture is unusual as it is the only PSP toxin producer reported to have lost the ability to produce toxins during routine culture maintenance over a prolonged interval (Martins et al. 2004). Since the identification of this non-toxic subculture, the strain has been characterized in several ways to determine the underlying mechanism for loss of toxicity.

One mechanism proposed to explain the loss of toxicity is that during culture maintenance, 18-1NT suffered a mutation that disrupted the biosynthetic pathway for PSP toxins. The interruption of the biochemical pathway leading to toxin production could imply that intermediate precursors, metabolites or degradation products will accumulate in the non-toxic *A. lusitanicum* cells, changing the biochemical composition, compared to the original 18-1 strain.

Consistent with this hypothesis, two peaks were detected in the 18-1NT HPLC analyses that are not present in the 18-1T subculture (Fig.1). Both peaks were present in only non-toxic 18-1NT cultures, and have not been seen in HPLC analysis of basic saxitoxin runs of other non-toxic *A. minutum* strains analyzed with the same methods and instrument (D Kulis, unpublished data). This argues against the unknown peaks corresponding to a cellular component common to *Alexandrium* or to 18-1 as, to our knowledge, the peaks are only present only in cell extracts of 18-1NT. Further characterization presented below indicates these compounds do not correspond to any of the known PSP toxins and evidence is provided that the compounds might be linked to the biosynthetic pathway of these toxins.

Chromatographic behavior

Peaks with a retention time fairly similar to the STX and neoSTX standards (designated as A11 and A12, respectively in Fig.1A and B) were observed in all HPLC analyses for cell extracts of 18-1NT. A11 has a retention time around 12 minutes, offset from the neoSTX standard by more than one minute. The A12 peak typically elutes at around 16 minutes, close but never coinciding with the STX standard in all HPLC runs.

Further experiments were performed to investigate the chemical nature of the A11 and A12 compounds and to rule out the possibility that A12 was saxitoxin. Both sample and standard (containing only STX) were incubated at 100°C in concentrated HCl (1:1 v/v) for 1 h. Though STX is considered fairly stable in acidic solutions, prolonged boiling is thought to hydrolyze the carbamoyl group of the STX molecule and convert it to dcSTX (Shimizu 2001). HPLC analysis of the concentrated, hot acid-treated samples showed different behaviors for the saxitoxin standard and A12 (Fig.2; Martins et al, 2004). For the hydrolyzed STX standard, the peak corresponding to STX disappeared and was replaced by a compound with a retention time equivalent to that of dcSTX. The 18-1NT A12 peak also disappeared, but did so without giving rise to a dcSTX-like compound. Unlike STX, A12 is an acid labile compound and a product of conversion was not found. However, the fact that both A11 and A12 peaks disappeared during acid hydrolysis does not rule out the possibility that the unknown compounds are PSP toxin congeners. While saxitoxin is converted into dcSTX after

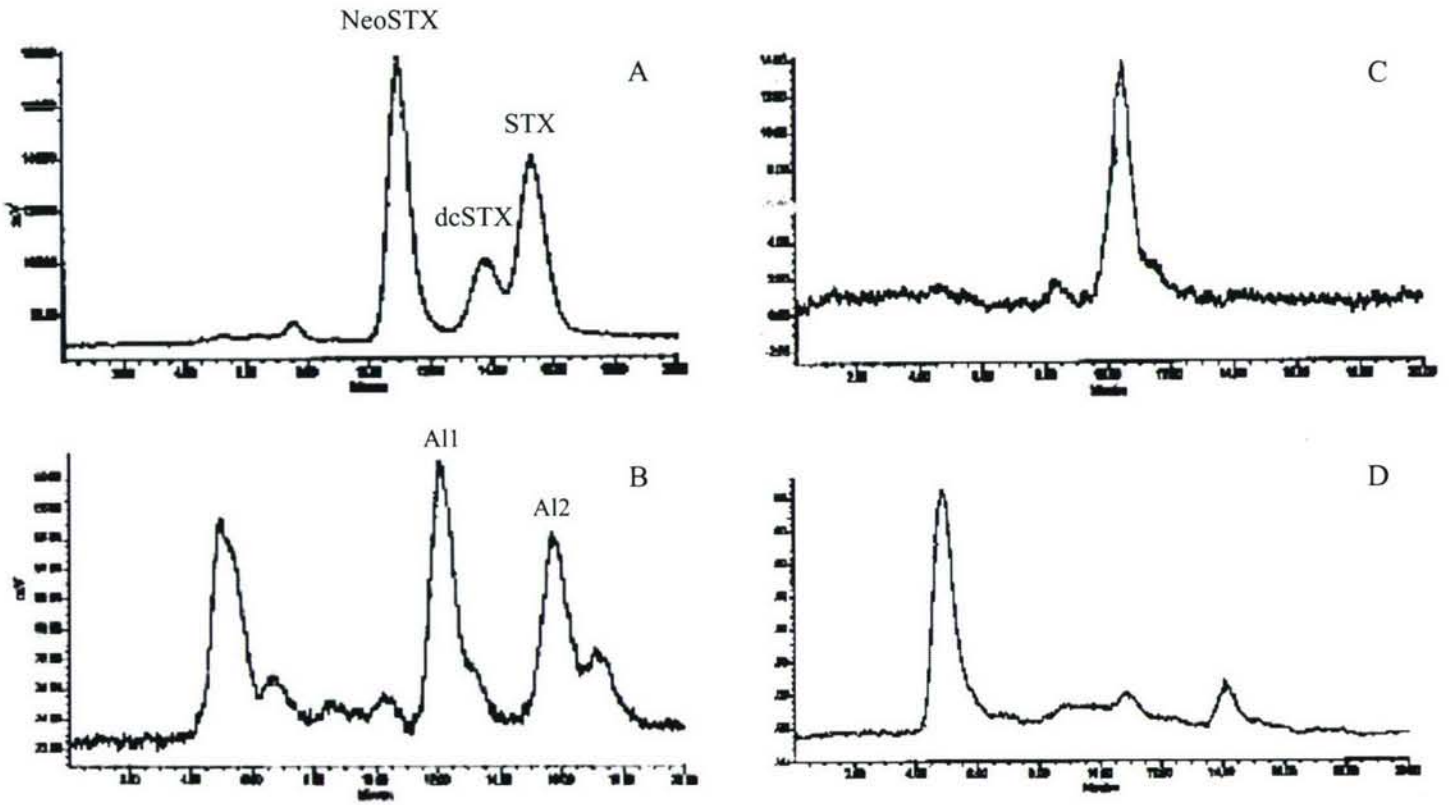

FIGURE 5-1. Representative chromatograms obtained using Oshima's post-column HPLC method for the detection of saxitoxin, dc-STX and neo-STX. Unknown peaks are labeled as Al1 and Al2. Top panels: standard mixture; bottom panels: 18-1NT cell extract. Samples were analyzed using fluorescent detection with (panels A and B) and without (panels C and D) oxidation of PSP toxins.

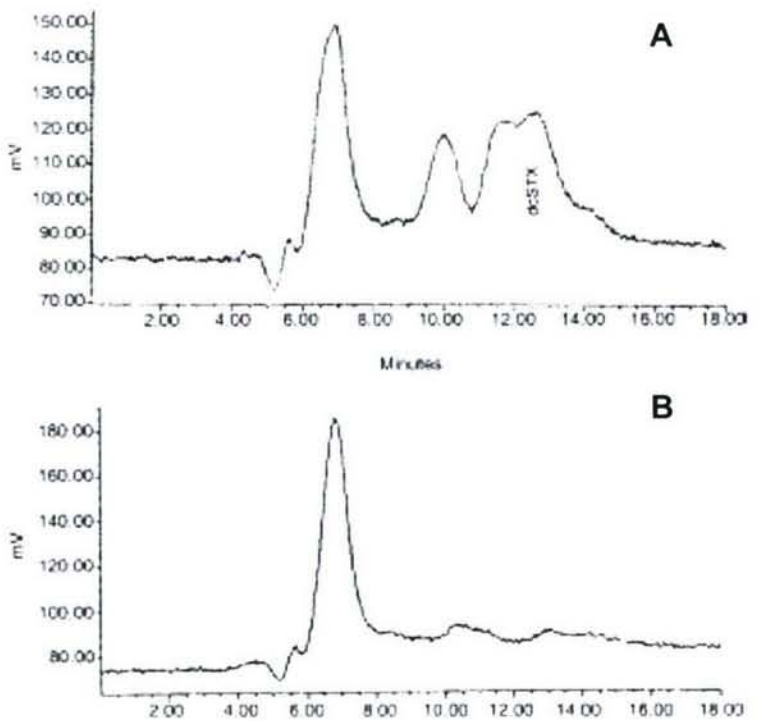

FIGURE 5-2. Chromatograms depicting the HPLC profiles obtained in samples analysed after one hour hydrolysis in HCl (1:1 v/v) at 100°C. Panel A shows the STX standard and panel B shows the sample extract (Martins et al, 2004).

heating in HCl, gonyautoxins and neo-STX are destroyed (Shimizu, 2001), much like A11 and A12.

Unknown sample components have been previously incorrectly identified as PSP toxins or peaks in chromatographic runs (Sato and Shimizu 1998). This is mainly due to the presence of naturally fluorescent compounds that elute at retention times close to those of PSP toxins. However, naturally fluorescent compounds can be distinguished from PSP toxins by omitting the post-column oxidizing step (Baker et al. 2003; Martins et al. 2003; Sato and Shimizu 1998). Briefly, according to the Oshima HPLC method, after chromatographic separation, the different toxin components are derivatized with an oxidizing reagent to fluorescent compounds. The oxidation reaction that turns saxitoxins into fluorescent compounds depends on the cleavage of the propiono ring structure of PSP toxins by the oxidant (Shimizu 2001). If the oxidant is replaced with distilled water, PSP toxins remain non-fluorescent and only naturally fluorescent compounds will be detected. The proposed chemical reaction occurring during post-column oxidation is depicted in Figure 3.

Figure 1 shows the product of the post-column oxidation reaction of standards and samples (panels A and B) as well as the chromatograms obtained for non-oxidized samples (panel C and D). Results show that, without the post-column oxidation, A11 and A12 behaved much like the standard toxin. However, A12 was greatly reduced after the post column oxidation process was deactivated, while the STX standard could not be detected. These results indicate that the unknown peaks are not naturally fluorescent compounds, but like PSP toxins become fluorescent after chemical oxidation. A similar structure may be present in the unknown compounds such that they undergo the same changes as saxitoxins in the presence of the oxidizing reagent.

The data presented thus far show that A11 and A12 are not known PSP toxins. However, the compounds may be previously undescribed saxitoxin congeners or compounds that share portions of their molecular structure with PSP toxins. If A11 and A12 are PSP toxin congeners, it is possible they might demonstrate biological toxicity and might be detected in biological assays aimed at detecting these effects.

Biological activity

To rule out the presence of toxic compounds, extracts of 18-1NT had been previously analyzed by three different toxin detection methods: HPLC, mouse neuroblastoma assay (MNA) and the mouse bioassay (MBA) (Martins et al. 2004). Both the MNA and the MBA provide information on toxin activity and the results of both methods showed that 18-1NT does not contain PSP toxins. These methods have an advantage over the HPLC, as they can detect a wide range of toxins by their biological activity and do not require the availability of standards for toxin identification. MNA is highly specific for sodium channel blocking toxins such as PSP toxins, while the MBA detects compounds with intra-peritoneal toxicity in mice. The MNA is the most sensitive of all the toxin detection methods used in this study (Gallacher and Birkbeck 1992). Despite this, MNA of 18-1NT extracts was negative for the presence of PSP toxins. Since saxitoxin and neoSTX have the highest toxicity of the known PSP toxins congeners (Oshima 1995), their presence should have been detected in the MBA. In summary, both MBA and MNA offer further evidence that A11 and A12 are

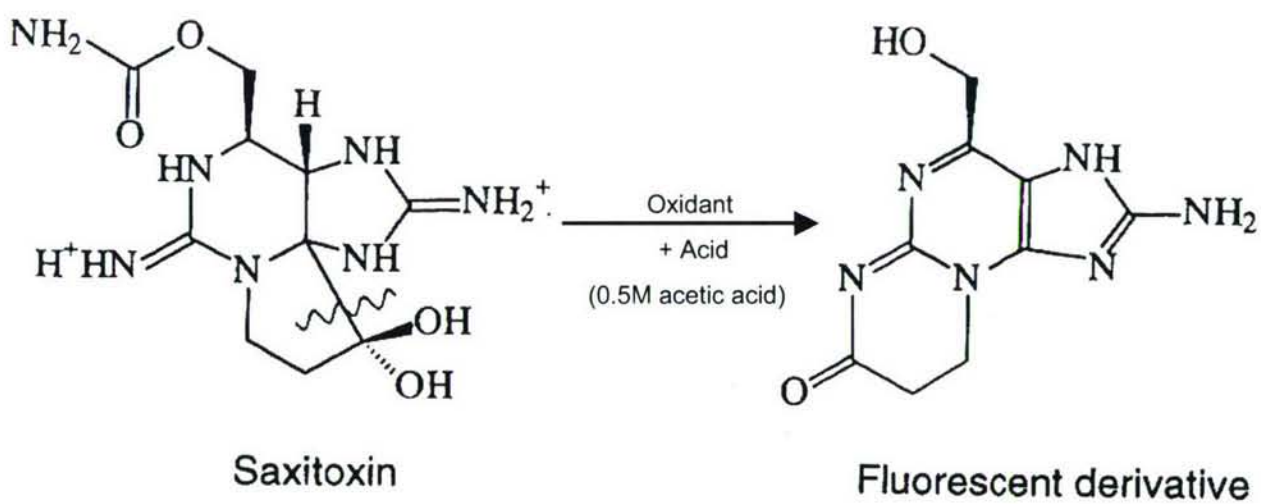

FIGURE 5-3. Formation of the fluorescent purine derivative as a result of the post-column oxidation of PSP toxins. Adapted from Boyer and Goddard (1999).

not STX and neoSTX as the unknown compounds lack sodium channel blocking activity and mouse toxicity.

Similar to conclusions from the previous section, the MBA and MNB results do not exclude the presence of a PSP toxin-related compound in 18-1NT extracts. Due to the nature of these biological tests, they would be unlikely to detect a saxitoxin congener with little to no biological activity. The toxicity of PSP toxins depends on the blockage of voltage-activated sodium channels on excitable cells. The degree of binding to sodium channels is contingent on the structure of the compound. PSP toxin congeners with very low levels of toxicity (Oshima, 1995) or no toxicity to mice (Onodera et al. 1997) have been previously identified. The presence of similar compounds in 18-1NT cannot be discounted by the MNA and MBA results.

Phosphate limitation

The nutrient content and to a lesser extent, the toxin profile, of dinoflagellates have been documented to change following nutrient limitation (Anderson et al. 1990b). Several authors have documented an increase in toxin content when cells are grown in P-limiting conditions (Anderson et al. 1990a; Bechemin et al. 1999; Boyer et al. 1987). Similar observations were made for cultures of 18-1T, the toxic variant of 18-1 (Table 1), during stationary growth phase (day 39) when the growth of phosphate deprived cultures was clearly limited (Fig.4).

When grown under P-limitation for 39 days, 18-1NT shows an increase in the per cell content of both the Al1 and Al2 compounds (Table 2). Considering the accumulation of certain toxin congeners under P-limitation (Anderson et al. 1990b; Taroncher-Oldenburg et al. 1999), the increase of Al1 and Al2 in P-limited conditions is consistent with the hypothesis that the unknown compounds are related to PSP toxin synthesis.

Alternatively, due to their behavior, Al1 and Al2 could represent cellular components related to cell stress and/or to a reduction in growth. The 18-1NT subculture has a slower growth rate and attains a lower maximum cell density than 18-1T (Martins et al. 2004). Cultures of 18-1NT during phosphate starvation have a further reduction in growth, reaching stationary phase earlier than phosphate replete cultures. Thus, the accumulation of Al1 and Al2 might be related to growth rather than toxin production, though the absence of natural fluorescence of the compounds and fluorescence with the oxidant makes this argument less likely. The strongest argument that these compounds are not related to growth stress is that they are not detected in phosphate limited 18-1T (Fig.5), though these cells are also under stress.

A similar experiment was performed where 18-1NT cells were grown under N-limitation. Previous work has demonstrated that the toxin content of *Alexandrium* decreases when kept under these conditions (Anderson et al, 1990b) and the same behavior was expected for Al1 and Al2 compounds, if these were related to PSP toxins. However, an N-limited 18-1NT culture could not be obtained in this study, as growth patterns of nutrient replete and N-deprived cultures were always comparable. Therefore, analysis of Al1 and Al2 content under N-limitation could not be performed. The reasons for the lack of differences in growth between N-replete and N-limited cultures are unknown. However, it is known nitrogen fixing bacteria can be

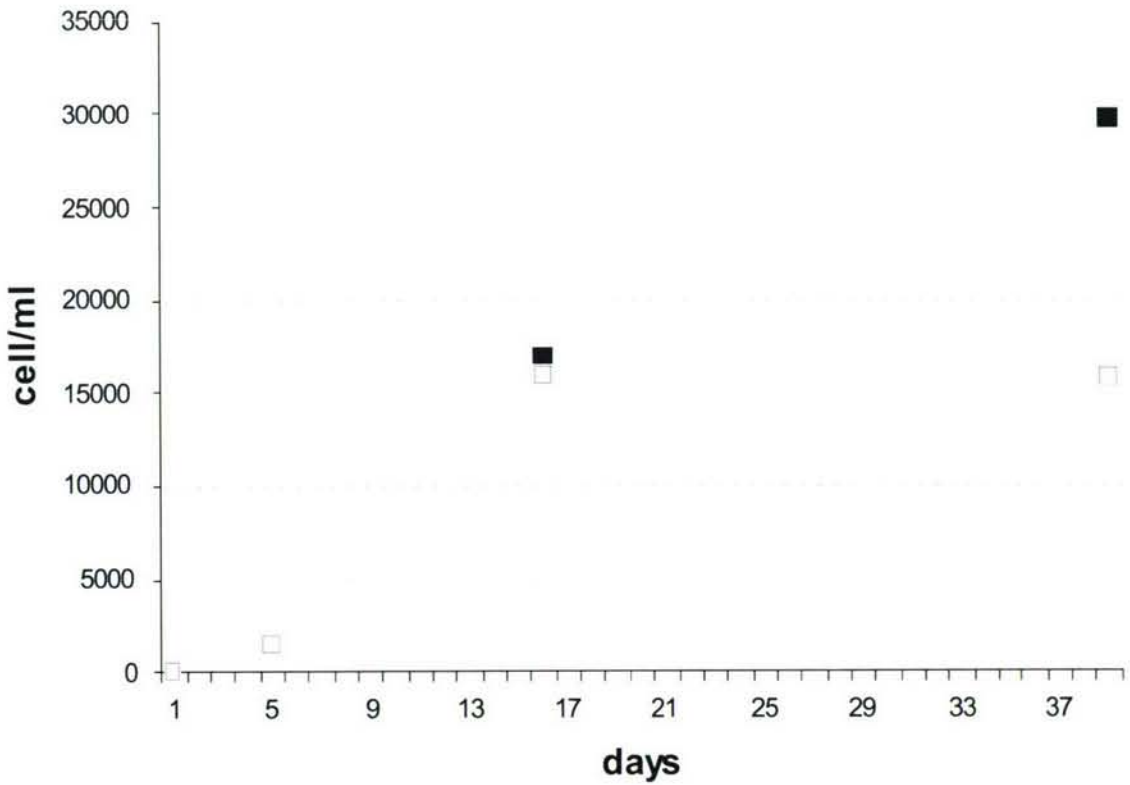

FIGURE 5-4. Cell counts obtained for 18-1T in lag (day 5), exponential (day 16) and stationary (day 39) phase of growth. Cultures were maintained in P-limiting (□) and nutrient replete conditions (■).

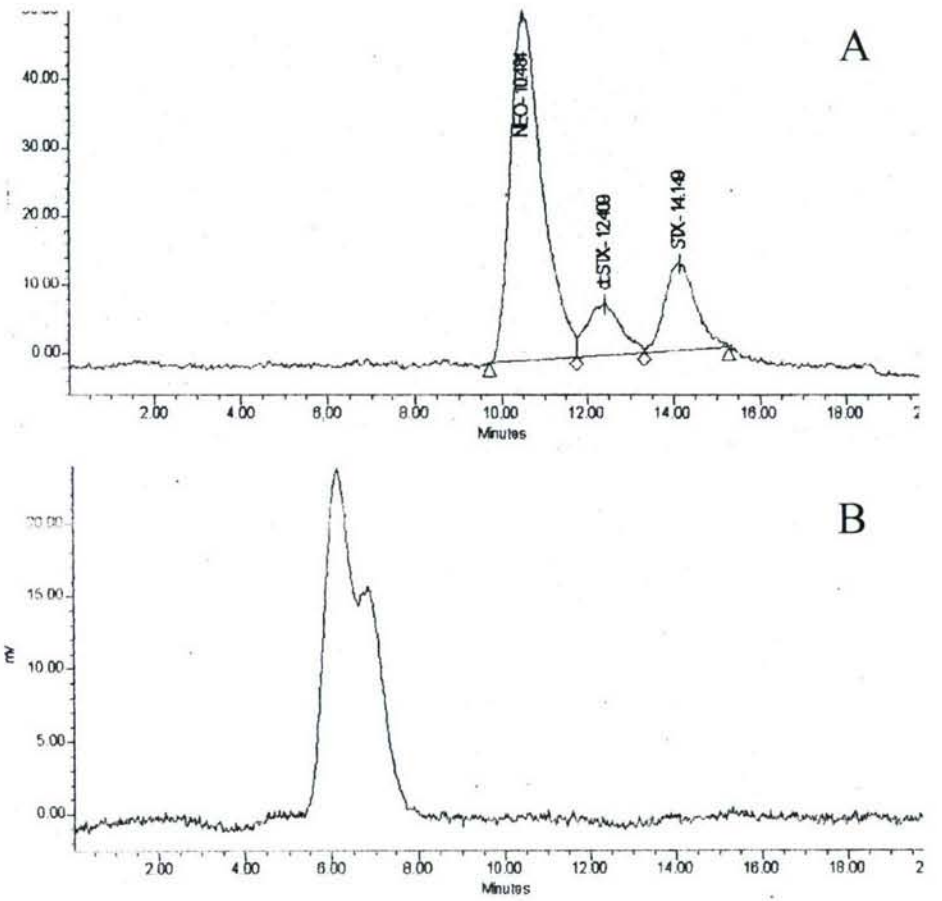

FIGURE 5-5. Representative chromatograms obtained using Oshima's post-column HPLC method for the detection of saxitoxin, dc-STX and neo-STX in cell extracts of 18-1T. Cultures of 18-1T were grown under P-limitation (panel B). Panel A depicts the standard mixture.

Table 5-1. Toxin content of 18-1T cultures grown in P-limiting and nutrient-replete conditions in each growth stage. Toxin measurements were performed in two replicate cultures for each growth condition (standard deviations when available are shown in parenthesis).

Growth phase	**Nutrient replete cultures (fmol/cell)**	**PO_3^- limited cultures (fmol/cell)**
Lag	10.1 (±0.87)	7.7
exponential	13.5 (±1.4)	11.0 (±1.0)
Stationary	9.6 (±0.30)	19.3 (±1.7)

found associated with algae (Martinez et al. 1983). It is possible nitrogen fixing bacteria might be present in 18-1 cultures, thus supplying the necessary nitrate to the dinoflagellate. No evidence for this hypothesis is currently available, as bacteria identified from 18-1 cultures do not seem to be involved in nitrogen fixation (Chapter 3).

Compound stability

It was difficult to calculate the concentration of All and Al2 compounds in 18-1NT cell extracts, as peak areas varied between different analyses of the same extract and their fluorescence quantum yields are unknown. The changes in peak area were hypothesized to be related to the stability of the unknown compounds. To investigate this, a controlled experiment was performed in which the same sample was analyzed twice, immediately after extraction and once again after storage at 4°C for one week. The observed increase in concentration of All with a concomitant decrease of Al2 (Table 3), during storage at 4°C, might indicate epimerization of the unknown compounds into a more stable form. PSP toxins have been previously shown to be stable at 4°C in acid solution (Louzao et al. 1994) though conversion among epimers can occur in extracts stored at these temperatures (Negri et al. 2003).

CONCLUSIONS

The structures of the All and Al2 peaks identified in this study remain unknown. Considerable evidence argues that All and Al2 are not known PSP toxins. The retention times of All and Al2 are sufficiently different from known toxins and biological assays did not detect any activity expected from most known PSP toxins. Though the retention time of Al2 is somewhat close to STX, this unknown compound behaves unlike the toxin standard in terms of stability under acid storage and does not convert to dcSTX when boiled in HCl.

The possibility that the compounds have chemical and physiological characteristics similar to PSP toxins cannot be excluded. All and Al2 accumulate in cells under phosphate stress much as PSP toxins do, and are also derivatized by post-column oxidation during HPLC. Considering these similarities, it is possible that All and Al2 could be previously unidentified PSP toxin congeners, whose occurrence is unique to the non-toxic form of 18-1. Alternatively, the unknown compounds detected in HPLC analysis could correspond to metabolites somehow linked to the toxin biosynthetic pathway. The lack of toxicity in the 18-1NT culture implies that an interruption in that pathway has occurred. In this scenario, metabolites or precursors would accumulate in the non-toxic *A. lusitanicum* cells, most likely the compounds produced in the step prior to the block. Another possibility is that All and Al2 are degradation products of the saxitoxin biochemical pathway. Regardless of whether All and Al2 are precursors, metabolites or degradation products, if this general line of thought is correct, a parsimonious explanation is that accumulation of these compounds is correlated not only to toxin production but to the reduction observed in the growth rate of 18-1NT and a failure of 18-1NT to reach high cell densities (Martins et al, 2004). The hypothesis that All and Al2 might correspond to intermediates in the PSP toxin biosynthesis and/ or be chemically related to saxitoxin will only be truly resolved with detailed structural work.

Table 5-2. Composition of unknown compounds A11 and A12, as determined by HPLC, in cells of *A. lusitanicum* 18-1NT grown under phosphate limitation or in replete cultures in f/2 medium. Measurements shown were obtained during stationary phase of both conditions. Concentrations are expressed in peak area due to the lack of suitable standards to quantify the compounds. The experiment was performed in duplicate (n=2) (standard deviations are shown in parenthesis).

Unknown compound	**Nutrient replete cultures (peak area/cell)**	**PO_3^- limited cultures (peak area/cell)**
A11	129,985 (± 3,220)	364,880 (± 54,281)
A12	101,776 (± 46,191)	176,815 (± 1,050)

Table 5-3. A11 and A12 composition expressed in per cent of total unknown compounds present in one 18-1NT extract. Cell pellets of 18-1NT were extracted in 0.5M acetic acid and analyzed by HPLC immediately (fresh extract). The remainder cell extract was stored at 4°C prior to second HPLC analysis (stored extract).

Unknown compound	**Fresh extract (% compound)**	**Stored extract (% compound)**
A11	62	42
A12	38	58

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REFERENCES

Anderson, D., D. Kulis, J. Sullivan, S. Hall, and C. Lee. 1990a. Dynamics and physiology of saxitoxin production by the dinoflagellates *Alexandrium* spp. Marine Biology **104:** 511-524.

Anderson, D. M., D. M. Kulis, G. J. Doucette, J. C. Gallagher, and E. Balech. 1994. Biogeography of toxic dinoflagellates in the genus *Alexandrium* from the northeastern United States and Canada. Marine Biology **120:** 467-478.

Anderson, D. M., D. M. Kulis, J. J. Sullivan, and S. Hall. 1990b. Toxin composition variations in one isolate of the dinoflagellate *Alexandrium fundyense*. Toxicon **28:** 885-893.

Baker, T. R., G. J. Doucette, C. Powell, G. L. Boyer, and F. G. Plumley. 2003. GTX4 imposters: characterization of fluorescent compounds synthesized by *Pseudomonas stutzeri* SF / PS and *Pseudomonas* / *Alteromonas* PTB-1, symbionts of saxitoxin-producing *Alexandrium* spp. Toxicon **41:** 339-347.

Bechemin, C., D. Grzebyk, F. Hachame, C. Hummert, and S. Y. Maestrini. 1999. Effect of different nitrogen/phosphorus nutrient ratios on the toxin content in *Alexandrium minutum*. Aquatic Microbial Ecology **20:** 157-165.

Boyer, G. L., J. J. Sullivan, R. J. Andersen, P. J. Harrison, and F. J. R. Taylor. 1987. Effects of nutrient limitation on toxin production and composition in the marine dinoflagellate *Protogonyaulax tamarensis*. Marine Biology **96:** 123-128.

Carmichael, W. W. 1994. Harmful cyanobacteria blooms, p. 166, Proceedings of the 37th Conference of the International Association of Great Lakes Research and Estuarine Research Federation, Windsor, ON (Canada), 5-9 Jun 1994. IAGLR.

Cembella, A. D. 1998. Ecophysiology and metabolism of paralytic shellfish toxins in marine microalgae, p. 381-403. *In* D. M. Anderson, A. D. Cembella and G. M. Hallegraeff [eds.], Physiological Ecology of Harmful Algal Blooms. NATO ASI Series. Springer-Verlag.

Cembella, A. D., J. J. Sullivan, G. L. Boyer, F. J. R. Taylor, and R. J. Andersen. 1987. Variation in paralytic shellfish toxin composition within the *Protogonyaulax tamarensis/catenella* species complex; red tide dinoflagellates. Biochemical Systematics and Ecology **15:** 171-186.

Franca, S. and others 1995. Prokaryotes in association with a toxic *Alexandrium lusitanicum* in culture, p. 45-51. *In* P. Gentien and C. Marcaillou-Le Baut [eds.], Harmful Marine Algal Blooms/Proliferation d'Algues Marines Nuisibles. Proceedings of the Sixth International Conference on Toxic Marine Phytoplankton, Nantes (France), Oct 1993. Lavoisier.

Gallacher, S., and T. H. Birkbeck. 1992. A tissue culture assay for the direct detection of sodium channel blocking toxins in bacterial culture supernates. FEMS Microbiology Letters **92:** 101-108.

Guillard, R. R. L., and J. H. Ryther. 1962. Studies of marine plankton diatoms I. *Cyclotella nana* Hustedt and *Detonula confervacea* (Cleve) Gran. Canadian Journal of Microbiology **8:** 229-239.

Louzao, M. C. and others 1994. Effect of lyophilization on the stability of gonyautoxins obtained from contaminated mussels. Toxicon **32:** 807-817.

Martinez, L., M. W. Silver, J. M. King, and A. L. Alldredge. 1983. Nitrogen fixation by floating diatom mats: a source of new nitrogen to oligotrophic ocean waters. Science **221:** 152-154.

Martins, C. A., P. Alvito, M. J. Tavares, P. Pereira, G. Doucette, and S. Franca. 2003. Reevaluation of production of paralytic shellfish toxin by bacteria associated with dinoflagellates of the Portuguese coast. Applied and Environmental Microbiology **69:** 5693-5698.

Martins, C. A., D. Kulis, S. Franca, and D. M. Anderson. 2004. The loss of PSP toxin production in a formerly toxic *Alexandrium lusitanicum* clone. Toxicon **43:** 195-205.

Mascarenhas, V., P. Alvito, S. Franca, I. Sousa, J. A. Rodriguez-Vasquez, and A. G. Martinez. 1995. The dinoflagellate *Alexandrium lusitanicum* isolated from the coast of Portugal: observations on toxicity and ultrastructure during growth phases, p. 71-76. *In* P. Lassus, G. Arzul, E. Erard-Le Denn, P. Gentien and C. Marcaillou-Le Baut [eds.], Harmful Marine Algal Blooms/Proliferation d'Algues Marines Nuisibles. Proceedings of the Sixth International Conference on Toxic Marine Phytoplankton, Nantes (France), Oct 1993. Lavoisier.

Negri, A. and others 2003. Three novel hydroxybenzoate saxitoxin analogues isolated from the dinoflagellate *Gymnodinium catenatum*. Chem. Res. Toxicol. **16:** 1029-1033.

Onodera, H., M. Satake, Y. Oshima, T. Yasumoto, and W. W. Carmichael. 1997. New saxitoxin analogues from the freshwater filamentous cyanobacterium *Lyngbya wollei*. Natural Toxins **5:** 146-151.

Oshima, Y. 1995. Post-column derivatization HPLC methods for paralytic shellfish poisons, p. 81-94. *In* G. M. Hallegraeff, D. M. Anderson and A. D. Cembella [eds.], Manual on harmful marine microalgae, IOC manuals and guides. UNESCO.

Pereira, P., D. Andrinolo, F. Sam-Bento, P. Alvito, C. Martins, and S. Franca. 2000. Novos resultados sobre estudos de toxicidade PSP em dinoflagelados e bacterias associadas, p. 139-148, VI Reunion Iberica sobre Fitoplancton Toxico y Biotoxinas. CAPDG, Junta de Andalucia.

Sato, S., and Y. Shimizu. 1998. Purification of a fluorescent product from the bacterium *Moraxella*: a neosaxitoxin impostor, p. 465-467. *In* B. Reguera, Blanco, J., Fernandez, M.L. and Wyatt, T. [ed.], Harmful Algae. Xunta de Galicia and IOC.

Schantz, E. J. 1984. Historical perspective on paralytic shellfish poison, p. 99-111. *In* E. P. Ragelis [ed.], Seafood Toxins. Based on a Symposium Sponsored by the Division of Agricultural and Food Chemistry at the 186th Meeting of the American Chemical Society, Washington D.C. August 28-September 2, 1983.

Shimizu, Y. 2001. Chemistry and mechanisms of action, p. 151-172. *In* L. Botana [ed.], Deafood and Freshwater Toxins. Marcel Dekker.

Taroncher-Oldenburg, G., D. M. Kulis, and D. M. Anderson. 1999. Coupling of Saxitoxin Biosynthesis to the G sub(1) Phase of the Cell Cycle in the Dinoflagellate *Alexandrium fundyense*: Temperature and Nutrient Effects. Natural Toxins **7:** 207-219.

Utermöhl, H. 1958. Neue Wege in der quantitativen Erfassung des Planktons (mit besonderer Berücksichtigung des Ultraplanktons. Verh. int. Ver. theor. angew. Limnol. **5:** 567-596.

CHAPTER 6

CONCLUSIONS

In the course of this study, new information was obtained concerning dinoflagellate toxin production and growth in culture. The fact that two subcultures of the same original isolate of *Alexandrium lusitanicum* 18-1 are clonal and one has completely lost toxicity demonstrated, for the first time, that the ability to produce saxitoxins in dinoflagellates of the genus *Alexandrium* can be lost.

It was crucial to this work to provide solid evidence that two *A. lusitanicum* subcultures were clones of the same parent culture and that they differ in their ability to produce saxitoxins. The fact that the cultures are clonal was first demonstrated by sequencing different regions of the nuclear ribosomal DNA. Additional experimental work has further supported the genetic similarity of both cultures through the use of microsatellites (L. McCauley, Anderson Laboratory, unpublished data) and random amplification of polymorphic DNA (RAPD) analysis of axenic cultures (data not shown). The total loss of toxin production was confirmed by 3 independent methods namely, two biological methods (mouse neuroblastoma assay and mouse bioassay) and one chemical method, high performance liquid chromatography (HPLC).

The existence of a non-toxic, slower-growing mutant of a toxic strain was a novel experimental system in which to study the mechanisms involved in the loss of toxin production and growth capability in dinoflagellates. It was hypothesized that the loss of toxicity in one of the subcultures as well as the observed differences in growth would be reflected in differences in gene expression and gene products of the two cultures. Because the two 18-1 cultures are very closely related and were maintained in similar growth conditions, the remaining genes expressed in each and their products should be highly shared. It was also hypothesized that the toxicity and growth differences could relate to the associated bacterial communities in these two cultures. The two subcultures were thus characterized in several ways to investigate these hypotheses.

Associated bacteria

Firstly, the potential role of bacteria in the loss of toxicity was addressed. Results showed that the bacterial assemblage of toxic and non-toxic subcultures of *A. lusitanicum* differed after years of separate culture maintenance and somewhat different culture conditions. However, the change in the associated microorganisms does not seem to have been the underlying cause of the observed differences in toxicity and growth capabilities, since adding bacteria from the toxic, faster growing culture to the non-toxic isolate did not elicit toxin production in the former, nor did it affect the reduced growth. These results are a convincing argument for the fact that dinoflagellates are the direct causative agents of saxitoxin production. It further supported the primary hypothesis that

changes in toxicity and growth were encoded in the dinoflagellate genes and/or their expression patterns.

In view of these results, proteome comparisons were performed (see below) using axenic subcultures. This approach excluded from the analysis all proteins from bacterial origin and/or proteins expressed by the dinoflagellate related to symbiotic interactions, as these are not related to the phenotypic differences observed in the two *Alexandrium* cultures.

Metabolome

HPLC analysis of cell pellets of the toxic and non-toxic *A. lusitanicum* cultures demonstrated that the two can be distinguished at the biochemical level, as two compounds were revealed to be present exclusively in the non-toxic subculture. These compounds were not any of the known saxitoxins, though they do share some structural and biochemical characteristics. This was expected as the loss of toxicity is likely to be related to a block in the saxitoxin biosynthetic pathway and therefore metabolites preceding the block could accumulate in the cells. The ultimate identity of these compounds remains unknown, as full elucidation of their structure would require isolation, purification and NMR analysis that were beyond the scope of this thesis. However, the similarities of the unknown HPLC peaks with PSP toxins warrants further studies aiming at elucidating their structure. Once the structures of the molecules are known, the biosynthetic pathway involved can potentially be deduced, establishing one more process which differs between toxic and non-toxic subcultures of *A. lusitanicum*.

Proteome

The study of the proteomes of both cultures allowed the identification of differentially regulated proteins that can be linked to dinoflagellate photosynthesis, specifically the Calvin cycle, and can be related to the reduced growth capability observed in the non-toxic subculture. The down regulation of enzymes involved in the Calvin cycle in the non-toxic subculture compared to the toxic one may help to explain the slower growth in the toxin-deficient clone of *A. lusitanicum*. However, it is unclear if the Calvin cycle reduction is a direct cause of the growth reduction or rather another consequence of an unidentified lesion, potentially somewhere in the photosynthetic apparatus of the non-toxic subculture, which is responsible for the reduction in growth. Some evidence collected suggest the latter might be the case, as the chlorophyll content was shown to be reduced in 18-1 non-toxic cells compared to its toxic counterpart.

Work performed in this thesis allowed the adaptation of a technique (DIGE) to dinoflagellates which can be applied to the study of a variety of cellular processes. The potential applications of the DIGE method are substantial including, for example, identification of both transcriptionally and translationally regulated genes in response to nutrient stress. Preliminary work demonstrated that 2-D gels can reveal a strong response to phosphate limitation at the protein level with several up-regulated proteins observed in the phosphate-limited proteome. Most importantly the proteomic results have demonstrated the usefulness of the growing number of dinoflagellate ESTs available. In

this particular study, the identification of some protein spots of interest was possible by comparing peptide mass fingerprinting data to the EST database of dinoflagellates and other organisms. Unfortunately, some proteins could still not be identified, highlighting the need for further genomic data for these organisms.

Though several differentially expressed proteins were identified through DIGE that cannot be linked to a specific function, their role in the saxitoxin pathway could not be confirmed. Proteins related to toxicity therefore might not have been identified with the DIGE analysis. This experimental system had some limitations that could explain these results. Specifically, if a point mutation (translated to a minor alteration at the protein level) was responsible for the loss of toxicity, it may not have been detected in a protein gel. Furthermore, gene products that are labile or occur in very low concentrations in the cell are also likely to have been missed by our experimental approach.

CONCLUSIONS

The mechanisms responsible for the initial loss of toxicity remain unknown, though the data acquired during this thesis leaves room for some speculation, particularly in view of the results presented in Appendix 1. The fact that a low level of toxicity and a higher growth rate were regained by the non-toxic 18-1 precludes the idea that a mutation was responsible for the differences in 18-1NT. A mutation reversal that would restore toxicity to the levels recorded in Appendix 1 seems an improbable occurrence. Therefore, it is considered more likely that a regulatory block was responsible for the loss of growth and toxicity in 18-1NT.

The fact that non-toxic cells grown in axenic conditions did not regain toxicity (Appendix 1) might suggest that bacteria have a role in PSP toxin production by dinoflagellates. The results in Appendix 1 support the argument that bacteria either autonomously produce low levels of toxins or are responsible for a part of PSP production by a dinoflagellate culture. However, both these arguments are inconsistent with the results of Chapter 3, as adding bacteria from the toxic culture did not cause 18-1NT to become toxic or regain growth. Furthermore, cultures maintained in ASP7 growth medium, in the presence of bacteria, also remained non-toxic and slower growing. Therefore, no evidence was found that can attribute the loss of toxicity and slower growth of 18-1NT to bacteria.

The mechanisms for the observed changes must lie elsewhere. The most parsimonious explanation is that the loss of toxicity and reduction in growth are not independent events. Evidence for this was also presented in Appendix 1, as cultures that regained some level of toxicity also demonstrate an improvement in growth.

The observations made in parallel to this thesis work regarding the 18-1NT photosynthetic apparatus may shed some light on the mechanisms explaining the reduction in growth. In fact, when 18-1NT cultures were visually compared to 18-1T, the non-toxic cultures typically appear lighter in color. When cell pellets of both cultures were analyzed for chlorophyll content, it was reported that 18-1NT had lower chlorophyll

content per cell in all growth stages (C. Gameiro, IO/FCUL, unpublished data). The low chlorophyll implies that the photosynthetic apparatus of 18-1NT differs from 18-1T. Since *A. lusitanicum* relies on photosynthesis for growth, it is likely this factor is the basis of the slower growth of the non-toxic subculture.

If it is accepted that all events are linked, it is feasible that the loss of toxicity and the loss of growth capability could be traced back to the photosynthetic apparatus and potentially the chloroplast. The idea that saxitoxin genes may somehow be related to the chloroplast is not completely implausible. Other than dinoflagellates, saxitoxin production has been demonstrated in more than one species of freshwater cyanobacteria (Lagos et al. 1999) and compelling evidence suggests that chloroplasts are derived from cyanobacteria (Tomitani et al. 1999). The theory proposed is that the saxitoxin genes were transferred to the dinoflagellate by its ancestral cyanobacteria symbiont and either remained in the reduced chloroplast genome or were transferred to the nucleus but encode proteins targeted to the chloroplast. The evidence for this theory so far is limited to the simultaneous observation that dinoflagellate cells where the photosynthetic apparatus has been affected, is both slower growing and has lost the ability to produce PSP toxins.

Future directions

It was demonstrated that DIGE can be a potent technique, potentially allowing the study of a variety of processes in dinoflagellates involving differential protein expression. Working specifically with the toxic and non-toxic clones, more information can be obtained in terms of proteins potentially linked to saxitoxin production. Only a few of the proteins identified as differentially expressed in DIGE were investigated. Furthermore, the use of different electrophoresis conditions such as narrower IPG strips or fractionation of protein extracts could potentially allow more proteins to be identified. DIGE can also be a useful tool in exploring other aspects of dinoflagellate metabolism, such as nutrient stress. This may lead to the identification of biomarkers of stress that could be very useful in determining the condition of cells in natural populations.

Furthermore, a powerful experimental system was identified that can undoubtedly provide information on the mechanisms involved in growth and toxin production. Very recent developments in the identification of genes involved in saxitoxin production have opened new lines of investigation that can use the full potential of the experimental system developed in this thesis. (Kellman and Nielan 2006 (oral presentation)) have used cyanobacteria as a model to identify saxitoxin genes. Cyanobacteria have significant advantages over dinoflagellates as model organisms as the saxitoxin genes are likely to be organized in operons and the genome is smaller. Using an approach that utilized the saxitoxin biosynthetic pathway predicted from Shimizu's studies (Shimizu et al. 1984) and chromosomal walking, these researchers were able to identify an operon that incorporated all predicted genes and was shown to be present only in saxitoxin-producing strains of cyanobacteria. A next step is to investigate how these results can be applied to dinoflagellates. These organisms may have acquired the "saxitoxin genes" separately or through horizontal gene transfer from cyanobacteria. In the latter case, genes acquired may or may not still remain as a cluster, but there may be significant sequence similarity.

The experimental model established in this thesis might be a good system in which to investigate the saxitoxin-related genes in dinoflagellates and, more importantly, understand the regulation and expression of toxicity. After some of the toxin genes have been identified in dinoflagellates, work could then focus on searching and characterizing these genes in both cultures of *A. lusitanicum* to understand which mutations might have occurred and their effects on saxitoxin biosynthesis. Alternatively, regulatory elements might have been affected in these cultures, which would be extremely useful information in order to understand the regulation of toxin production.

The study by Kellman and co-workers has also revised and modified the saxitoxin biosynthetic pathway proposed by Shimizu (1984) according to their results. If the structure of the unknown compounds identified in this thesis as present only in the non-toxic isolate was known, it would be interesting to establish if the compounds could fit in the new biosynthetic pathway.

The identification of saxitoxin-related genes in dinoflagellates would open many lines of investigation. For one, it would help to elucidate the mechanisms underlying observations made in this thesis that at present have no explanation. Such is the case of the effects of culture medium composition on the long-term loss or recovery of toxicity in the non-toxic *Alexandrium* subculture (Appendix 1). Since it is possible to elicit toxin production in the non-toxic culture after prolonged maintenance in f/2 medium, this seems to indicate the toxin genes described above are shut down rather than mutated. This hypothesis can only be tested if the toxin genes are identified.

Another question left unanswered is the role of bacteria on toxin production. It is clear bacterial symbionts are not the main factor affecting dinoflagellate toxicity. However, axenic cultures do seem to produce less toxin than their bacterized counterparts. This argues for a role of bacteria in indirectly regulating the saxitoxin pathway. It would be interesting to identify the molecular mechanisms of these effects, thus elucidating the symbiotic interaction at the gene level.

The toxic and non-toxic *A. lusitanicum* subcultures identified in this thesis have great potential to support many other studies addressing toxin production and growth by dinoflagellates. Future investigations will hopefully be able to build on from the work reported in this thesis.

REFERENCES

Kellman, and Nielan. 2006 (oral presentation). 12th International Conference on Harmful Algae.

Lagos, N., H. Onodera, P. A. Zagatto, D. Andrinolo, S. Azevedo, and Y. Oshima. 1999. The first evidence of paralytic shellfish toxins in the freshwater cyanobacterium *Cylindrospermopsis raciborskii*, isolated from Brazil. Toxicon **37:** 1359-1373.

Shimizu, Y., M. Norte, A. Hori, G. Almoursi, and M. Kobayashi. 1984. Biosynthesis of saxitoxin analogues: the unexpected pathway. Journal of the American Chemical Society **106:** 6433-6434.

Tomitani, A., K. Okada, H. Miyashita, H. Matthijs, T. Ohno, and A. Tanaka. 1999. Chlorophyll b and phycobilins in the common ancestor of cyanobacteria and chloroplasts. Nature **400:** 159-162.

APPENDIX 1

TOXIN PRODUCTION OF AN UNUSUAL *ALEXANDRIUM LUSITANICUM* CULTURE IN DIFFERENT GROWTH CONDITIONS

INTRODUCTION

Paralytic shellfish poisoning (PSP) is a potentially life threatening syndrome reported worldwide. Several species of dinoflagellates in the genus *Alexandrium* have been demonstrated to produce saxitoxins, a family of potent neurotoxins responsible for the symptoms associated with PSP intoxications. Numerous *Alexandrium* isolates from several worldwide locations have been characterized with respect to the amount and suite of toxins produced.

Toxin production is generally considered a constitutive characteristic of a particular culture of a dinoflagellate. Limited variations in toxin content and composition of a culture are observed, typically following sustained nutrient deprivation and adaptation (reviewed in (Anderson 1990); (Franco et al. 1995) or after prolonged culturing (Hansen et al. 2003). These variations reflect differences in a strain's growth conditions such as nitrate and phosphate concentrations (Anderson et al. 1990b) or salinity (Flynn et al. 1996).

Recently, the apparent constitutive character of toxin production was challenged by the identification of a culture of *Alexandrium* that had completely lost the ability to produce saxitoxins (Martins et al 2004). This culture of *A. lusitanicum* was isolated from Obidos Lagoon, Portugal, in 1962 and maintained in the laboratory in artificial seawater ASP7 medium. As routine procedure, to avoid bacteria contamination, cultures were treated with antibiotic mixture AM9 every two transfers (Silva and Sousa 1981). In 1992, a subculture of this isolate was established and maintained as a separate isolate in the seawater base medium, f/2 (Guillard and Ryther 1962) with no further antibiotic addition. In 2000, toxin analysis (Martins et al, 2004) showed that while the variant in f/2 had the same toxin content and toxin composition as the initial culture, the culture treated with antibiotics and maintained in ASP7 no longer produced detectable toxin levels. Due to the observed differences in toxicity, the toxic subculture was renamed 18-1T and the non-toxic isolate 18-1NT. This was the first report of two clones from the same culture differing in their ability to produce toxins. In conjunction with the observations on toxicity, it was determined that the growth rate and maximum cell density of 18-1NT were significantly lower compared to the toxic isolate.

The existence of a toxin–deficient, slower-growing mutant is an ideal experimental system in which to study the mechanisms involved in the loss of toxin production and slower growth. In view of the knowledge of the culture's history, several mechanisms were proposed to explain the observed changes in 18-1NT. One striking difference

between the culture conditions of 18-1T and 18-1NT was the sustained use of antibiotics in the non-toxic isolate every two transfers. Antibiotics are likely to have affected the growth and viability of specific bacteria associated with 18-1NT and the probable changes in the bacterial community could be the underlying cause of the loss of toxicity in this subculture.

To investigate this hypothesis, 18-1NT was grown with its own bacterial assemblage plus the assemblage of 18-1T. If in fact the loss of specific bacteria was responsible for the lack of toxicity in 18-1NT, then adding bacteria from 18-1T should restore the phenotype. The experimental design reported here was somewhat complementary to that described in previous work (Chapter 3).

MATERIALS AND METHODS

Dinoflagellate cultures

Cultures of 18-1T and 18-1NT were obtained as described previously (Martins et al. 2004). To ensure that cultures were maintained in the same conditions, 18-1NT, previously maintained in ASP7 with periodic addition of antibiotics (Silva and Sousa 1981), was transferred to f/2 medium. The culture was maintained in this growth medium for over 3 years before the experimental work was performed.

Cross-inoculation

Cells of 18-1NT were inoculated into medium composed of equal parts of spent growth medium from the 18-1T culture and sterile f/2. Spent medium was defined as f/2 growth medium in which 18-1T cultures were maintained through exponential phase, at which time dinoflagellates in the culture were disrupted and then removed by filtration. Dinoflagellates were lysed by briefly vortex mixing a 15 ml aliquot of culture with glass beads in order to release attached and intracellular bacteria. The resulting lysate was filtered through a 5 μm pore size filter (Millipore), to ensure removal of dinoflagellate cells and cell debris, while allowing for the passage of free bacteria. Cultures thus inoculated were maintained for 5 months before measurements were taken. Controls for this experiment included the inoculation of spent media in f/2 medium to test for the presence of viable cells and 18-1NT cultures grown in f/2 media not supplemented with spent media.

Growth of cross-inoculated cultures was monitored through in vivo fluorescence in a Turner Designs Model 10 fluorometer. Cells were harvested periodically for toxin measurements and cell counts in a Sedgwick-Rafter chamber (see below). The growth and toxin production of 18-1NT cultures thus obtained was further compared to axenic cultures of 18-1NT obtained in a parallel study (Chapter 3).

Toxin analysis

Prior to harvesting cells for toxin analysis, two 1.5 ml aliquots were removed from well mixed 18-1NT cultures and fixed in Utermöhl's solution (Utermöhl 1958) for counts using a Sedgwick-Rafter counting chamber. Depending on cell density, the whole counting chamber or at least 400 cells were counted. An average of the two aliquots was calculated and this value reported as the cell count for the culture.

For toxin extraction, 15 ml of cultures were collected by centrifugation (5000g; 5 min, 23°C) and the resultant cell pellets resuspended in 1ml of 0.05M acetic acid. The acetic acid cell slurry was then disrupted by sonication (10 W, 3 cycles of 10 s.) on ice. Samples were frozen at -20°C prior to analysis. All toxin analyses were performed by the HPLC method of (Oshima 1995), with slight modifications as described in (Anderson et al. 1994).

Four mixtures of PSP toxin standards kindly provided by Prof. Oshima (Tohoku University, Sendai, Japan) were used for determining the toxin profile and toxin content. Toxicities of the derivatives (in fg STX equivalents.cell^{-1}) were calculated from the molar composition data using individual potencies in mouse units per mmol^{-1} (Oshima, 1995).

RESULTS AND DISCUSSION

In several experiments performed from 2000 to 2004, the 18-1NT culture was consistently noted to produce no saxitoxins, despite having been isolated originally as a toxic dinoflagellate culture. The total loss of toxin production of a previously toxic *Alexandrium* had never been documented and thus challenged the paradigm that the ability to produce saxitoxins is a constitutive characteristic of a species. The fact that a subculture of the original *A. lusitanicum* culture exists that maintains the same toxin profile (composition) and roughly the same toxin content as previously described for the parental isolate made this pair of cultures an ideal experiment to study the mechanisms responsible for the loss of toxin production.

One main difference between the two *A. lusitanicum* subcultures was the sustained use of antibiotics in the 18-1NT culture. In all likelihood, certain species of bacteria will be more susceptible to the antibiotic mixture than others, potentially altering the existing bacterial assemblage of an organism. This change in the associated bacteria of 18-1NT was hypothesized as being the underling factor in the loss of toxicity in the 18-1NT culture, as well as the observed reduction in growth capability.

The effect of specific bacteria on dinoflagellate toxin production had been previously suggested (Silva 1962), with a number of reports providing evidence for this. For example, the removal of bacteria had been demonstrated to decrease toxicity to 1/5 of the level in the xenic culture (Uribe and Espejo 2003) in a strain of *Alexandrium*. Bacteria also have been shown to affect dinoflagellate growth. Certain bacteria are algicidal and have a serious negative effect on dinoflagellate viability (Doucette et al. 1999) while others seem to promote growth (Doucette 1995). Axenic cultures have also been shown to have decreased growth capabilities (Hold et al. 2001).

The potential bacterial involvement in the loss of toxicity of 18-1NT gained further support when it was observed that the bacterial assemblage of 18-1T and 18-1NT differed, as concluded from comparison of DGGE patterns (Chapter 3). In view of these results, multiple approaches were devised with the ultimate aim that the bacteria associated with the toxic strain were transferred to the non-toxic subculture. This subculture was then analyzed through time for any changes in toxicity and growth capabilities.

Experiments performed included growing both cultures in the absence of bacteria or in contact with each other through a 5 μm filter to allow free exchange of bacteria (Chapter 3). Here I present the results obtained when spent medium from 18-1T cultures, containing bacteria from this dinoflagellate, was added to cultures of 18-1NT. In other words, the bacterial assemblage of this cross-inoculated 18-1NT will include its own bacteria plus the bacteria from 18-1T.

In these experiments, small traces of GTX1 and GTX 4 were recorded for cultures of the 18-1NT cross-inoculated culture. The reduction in growth capability which had previously been described for 18-1NT was also observed to be altered by the addition of spent medium, as the cross-inoculated culture now exhibited the same growth pattern as the 18-1T faster growing subculture. Similar observations were also made for the 18-1NT control cultures. Control cultures of 18-1NT had been kept in sterile f/2 medium only. Detailed results are presented below.

Toxicity

Previous experiments (Chapter 3) showed bacteria are not involved in the loss of saxitoxin production, as axenic cultures continued to produce toxins, though at a lower level. Similarly, no changes in the toxin production were reported for cultures maintained in mixing chambers.

The cross-inoculation experiments however, seemed to contradict these results. On addition of 18-1T spent media, cross-inoculated cultures were monitored regularly by in vivo fluorescence measurements. When cultures reached exponential phase, aliquots of the culture were removed for toxin determination. HPLC analysis revealed that the 18-1NT culture to which 18-1T spent medium had been added contained trace amounts of GTX1 and GTX4 (Fig.1). These peaks were identified as PSP toxins due to the retention time matching the standards and similar behavior when the oxidant is replaced with distilled water in HPLC runs. The same toxin peaks were latter observed in the control 18-1NT cultures kept in f/2 medium, ruling out any role of the 18-1T spent media in eliciting toxin production in 18-1NT. These observations were surprising, as 18-1NT was non-toxic in all previous measurements (Martins et al, 2004).

The toxin epimers now detected in 18-1NT are the main toxin component of the toxic 18-1T subculture. This might indicate 18-1NT is reverting to its original toxic form. However, when compared to the toxic 18-1T, 18-1NT had substantially lower toxin

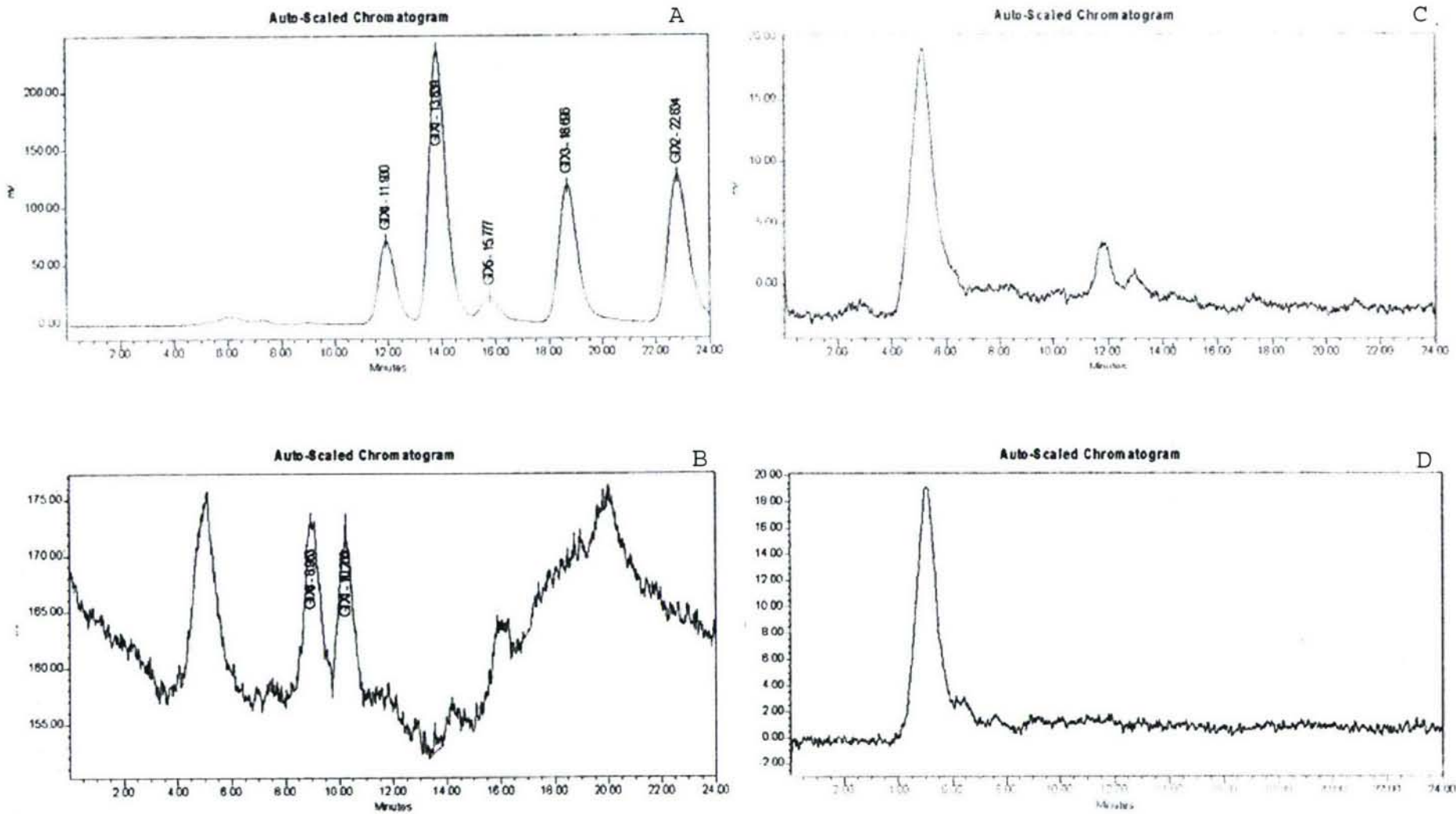

FIGURE A-1. HPLC chromatograms representing typical toxin profiles obtained for each 18-1NT subculture. A: GTX standard; B: toxin profile obtained for 18-1NT cultures maintained in f/2 medium supplemented with 18-1T spent medium (see material and methods for details) (separate run); C: toxin profile of control 18-1NT cultures grown in sterile f/2 medium since 2000; D: toxin profile of the original 18-1NT culture grown in ASP7 medium since 1962. Note y-axis have different scales.

content. During exponential stage the per cell toxin content for 18-1T was typically around 11.52 ± 1.99 fmol/cell (obtained in one set of experiments) while the toxin content for 18-1NT in one cell pellet was around 0.02 fmol/cell. It should be noted that quantification by HPLC at the lower of these levels is not accurate and is most certainly out of the linearity range of the method. However, the conclusion to be drawn is that 18-1T is 2 to 3 orders of magnitude or more toxic than 18-1NT. The lower toxicity of 18-1NT seems to indicate the saxitoxin biosynthetic pathway is still somehow blocked in this dinoflagellate, even though some toxin is being produced.

Growth

In addition to toxin observation, the growth of all cultures in this experiment was recorded by in vivo fluorescence. At the start of the experimental work, 18-1T was a faster growing culture, while 18-1NT had a lower growth rate. The measurements revealed that 18-1NT grown with spent medium had a growth pattern similar to 18-1T. Growth rates for 18-1NT plus spent media were on the order of $0.26d^{-1}$, compared to a growth rate of $0.23d^{-1}$ for 18-1T, obtained in previous experiments. The observed growth rate for 18-1NT in spent medium might indicate that bacteria have indeed increased the growth capacity of the non-toxic 18-1NT. However, the 18-1NT controls for this experiment, grown in f/2 medium only, were observed to have the same increase in growth. In other words, the increase of growth capability observed in 18-1NT cannot be attributed to the addition of 18-1T spent medium.

There are several potential explanations for the appearance of GTX1,4 and increase in growth rate in the non-toxic clone under spent medium and f/2 conditions: contamination with toxic 18-1T cells, the effect of different growth media and the effect of the bacterial assemblage.

Contamination with toxic cells

One alternative to explain the change in toxin production was that somehow the 18-1NT subculture had been contaminated with a few cells of the toxic culture 18-1T. This could certainly be a possibility for the 18-1NT culture to which 18-1T spent medium had been added, as 18-1T cells could have passed through the 5 μm filter.

However, during the course of these experiments, 18-1T spent media filtered through a 5 μm filter failed to yield viable dinoflagellate cells when inoculated in sterile f/2 medium. Other filter sizes, namely 13 μm, despite having a pore size substantially smaller than 18-1 cell diameter (20 μm), frequently let whole dinoflagellates through during filtration and 18-1T cells were readily visible under a magnifying lens after a few days of maintenance in f/2 medium.

Furthermore, the fact that the control 18-1NT cultures showed similar trace levels of toxin seems to indicate contamination was not an issue, as control cultures were maintained in sterile f/2 only and were handled separately. In addition, a subculture of 18-1NT in f/2 maintained at the laboratory of Dr. Susana Franca (LME, Portugal) was

also shown to contain traces of GTX1 and 4. It is unlikely similar levels of contamination with 18-1T cells had occurred in two different laboratories.

Growth medium

As discussed above all cultures maintained in f/2, with or without spent medium, show trace amounts of toxins. Fortunately, Dr. Franca kept a subculture of 18-1NT in ASP7, the artificial seawater media in which the original 18-1 culture had been isolated and maintained since 1962. No traces of saxitoxins could be detected in this subculture after several HPLC analyses in Dr. Franca's laboratory and our own (Fig.1, D).

The reason behind 18-1NT regaining toxicity after being transferred to and maintained in f/2 for a few years is unknown, as are the mechanisms responsible for the lack of the ability of the same culture to produce saxitoxins when maintained in ASP7. The lower toxicity of dinoflagellate cultures maintained in ASP7 has been shown previously (Reguera et al. 1993) and (Martins et al. 2004). The mechanisms are not understood and it has been suggested that a lack of certain nutrients in the artificial medium or the lower nutrient concentrations when compared to most seawater based media might be involved.

A decrease in toxicity of certain dinoflagellate cultures under certain types of nutrient stress has been previously documented. Cultures of the dinoflagellate *Alexandrium* have been extensively demonstrated to have a lower toxin content when grown under nitrate limitation (e.g. (Anderson et al. 1990a; Bechemin et al. 1999). However, despite these known variations, toxin production has never been elicited in non-toxic dinoflagellate cultures kept in nutrient rich conditions. In the same way, the total loss of toxicity in limiting conditions has never been observed.

However, experiments addressing the effects of long term maintenance of dinoflagellate cultures in nutrient rich or nutrient deficient conditions are lacking, and most data available reflect short term experimental work, over the course of a few culture transfers. 18-1NT has been kept in ASP7 for over 40 years, and it is possible the lack of a certain nutrient in this artificial-seawater medium or the lower levels of nutrients might have blocked the saxitoxin biosynthetic pathway. This block could potentially be partially removed after long term maintenance in f/2 medium. 18-1NT remained non-toxic in f/2 medium from 2000 to 2004. This suggests that significant time is required for toxin production to be regained after cultures are transferred to a different medium.

One final comment to these observations is the link between toxicity and maintenance in culture. Several authors have documented changes in the toxin content of cultures kept for extended periods of time. These changes range from substantial reductions in the toxin content (Hansen et al. 2003), to increase in toxicity (Bechemin et al. 1999) to no changes at all (D Anderson, unpub data). While these reports are sporadically found in the literature or personal communications, to our knowledge no attempt has been made to understand the mechanisms involved. The task is daunting and results could take years to obtain.

Bacterial assemblage

Results to date demonstrate that the addition of spent medium to 18-1NT was not responsible for the trace amounts of toxin observed in these cultures. This is because 18-1NT control cultures in only f/2 exhibited the same behavior. However, there are some arguments for some role of bacteria in dinoflagellate toxicity. Interestingly, no toxin peaks could be detected for axenic cultures of 18-1NT in any stage of the growth curve even though the base growth medium was f/2. We can only speculate why the absence of bacteria in the 18-1NT f/2 growth medium seems to have the same effect as maintaining the culture in ASP7. However, 18-1T cultures produce toxin in ASP7, f/2 and f/2 without bacteria, though in this last case, toxin content is reduced. One possible explanation for the lack of toxins in axenic 18-1NT in f/2 is therefore that toxicity has been reduced to levels that cannot be detected by HPLC.

Alternatively, it is possible the axenic 18-1NT is missing a compound essential to toxin production that is only found in the presence of f/2 medium and certain bacteria. Several authors have speculated that bacteria might influence dinoflagellate toxin production indirectly by providing required metabolites (eg. (Doucette 1995). This would reflect a symbiotic interaction for which evidence cannot be provided at this point.

Note that such an event has been described before in experiments attempting to elicit toxicity in non-toxic dinoflagellates by adding bacteria from toxic species. (Dimanlig and Taylor 1985) performed cross-inoculation experiments by adding 10-day old cultures of toxic *Protogonyaulax tamarensis* (=*Alexandrium tamarense*) filtered through 5 µm to non-toxic cultures of *P. tamarensis*. The authors observed trace toxin peaks in some cultures, but also in the control cultures (non-toxic *P. tamarensis* inoculated with spent media of toxic *P. tamarensis*). It was suggested the trace peaks might correspond to saxitoxin impostors, but no further confirmation was presented.

CONCLUSIONS

18-1NT is a unique dinoflagellate culture. It was the first saxitoxin producing dinoflagellate culture to have lost the ability to produce toxins. However, under certain conditions, 18-1NT can regain some toxicity, as trace amounts of GTX1,4 were detected after prolonged growth in a rich, seawater-based culture medium in the presence of bacteria. When grown in the absence of a bacterial assemblage or in the artificial-seawater medium ASP7, 18-1NT remains non-toxic. The mechanism for toxin suppression after prolonged maintenance in ASP7 is thus far unknown and cannot be easily tested due to the level of complexity involved with differences in the two growth media. However, the observations of toxin loss and then recovery after prolonged culturing in certain types of media suggest that there is an essential element missing under some culture conditions that can directly affect toxicity. Whether this might also happen during repeated population cycles in certain types of natural habitats remains to be seen.

It is interesting that the return of low levels of toxicity in 18-1NT seems to be associated with restoring growth capability. This seems to indicate that the alterations observed in 18-1NT growth and toxicity, may have been caused by the same event in 18-1NT, as both were restored simultaneously.

REFERENCES

Anderson, D., D. Kulis, J. Sullivan, S. Hall, and C. Lee. 1990a. Dynamics and physiology of saxitoxin production by the dinoflagellates *Alexandrium* spp. Marine Biology **104:** 511-524.

Anderson, D. M. 1990. Toxin variability in *Alexandrium* species., p. 41-51. *In* E. Granéli, B. Sundström, L. Edler and D. M. Anderson [eds.], Toxic Marine Phytoplankton. Elsevier, Inc.

Anderson, D. M., D. M. Kulis, G. J. Doucette, J. C. Gallagher, and E. Balech. 1994. Biogeography of toxic dinoflagellates in the genus *Alexandrium* from the northeastern United States and Canada. Marine Biology **120:** 467-478.

Anderson, D. M., D. M. Kulis, J. J. Sullivan, S. Hall, and C. Lee. 1990b. Dynamics and physiology of saxitoxin production by the dinoflagellates *Alexandrium* spp. Marine biology **104:** 511-524.

Bechemin, C., D. Grzebyk, F. Hachame, C. Hummert, and S. Y. Maestrini. 1999. Effect of different nitrogen/phosphorus nutrient ratios on the toxin content in *Alexandrium minutum*. Aquatic Microbial Ecology **20:** 157-165.

Dimanlig, M. N. V., and F. J. R. Taylor. 1985. Extracellular bacteria and toxin production in *Protogonyaulax species*, p. 103-108. *In* D. M. Anderson, A. W. White and D. G. Baden [eds.], Toxic Dinoflagellates. Proceedings of the Third International Conference on Toxic Dinoflagellates, St. Andrews, N.B. (Canada), 8-12 Jun 1985. Elsevier.

Doucette, G. J. 1995. Interactions between bacteria and harmful algae: A review. Natural Toxins **3:** 65-74.

Doucette, G. J., E. R. McGovern, and J. A. Babinchak. 1999. Algicidal bacteria active against *Gymnodinium breve* (Dinophyceae). I. Bacterial isolation and characterization of killing activity. Journal of Phycology **35:** 1447-1454.

Flynn, K. J., K. Flynn, E. H. John, B. Reguera, M. I. Reyero, and J. M. Franco. 1996. Changes in toxins, intracellular and dissolved free amino acids of the toxic dinoflagellate *Gymnodinium catenatum* in response to changes in inorganic nutrients and salinity. Journal of Plankton Research **18:** 2093-2111.

Franco, J. M., S. Fraga, M. Zapata, I. Bravo, P. Fernandez, and I. Ramilo. 1995. Comparison between different strains of genus *Alexandrium* of the minutum group, p. 53-58. *In* P. Lassus, G. Arzul, E. Erard-Le Denn, P. Gentien and C. Marcaillou-Le Baut [eds.], Harmful Marine Algal Blooms/Proliferation d'Algues Marines Nuisibles. Proceedings of the Sixth International Conference on Toxic Marine Phytoplankton, Nantes (France), Oct 1993. Lavoisier.

Guillard, R. R. L., and J. H. Ryther. 1962. Studies of marine plankton diatoms I. *Cyclotella nana* Hustedt and *Detonula confervacea* (Cleve) Gran. Canadian Journal of Microbiology **8:** 229-239.

Hansen, G., N. Daugbjerg, and J. M. Franco. 2003. Morphology, toxin composition and LSU rDNA phylogeny of *Alexandrium minutum* (Dinophyceae) from Denmark, with some morphological observations on other European strains. Harmful Algae **2:** 317-335.

Hold, G. L., E. A. Smith, T. H. Birkbeck, and S. Gallacher. 2001. Comparison of paralytic shellfish toxin (PST) production by the dinoflagellates *Alexandrium lusitanicum* NEPCC 253 and *Alexandrium tamarense* NEPCC 407 in the presence and absence of bacteria. FEMS Microbiology Ecology **36:** 223-234.

Martins, C. A., D. Kulis, S. Franca, and D. M. Anderson. 2004. The loss of PSP toxin production in a formerly toxic *Alexandrium lusitanicum* clone. Toxicon **43:** 195-205.

Oshima, Y. 1995. Post-column derivatization HPLC methods for paralytic shellfish poisons, p. 81-94. *In* G. M. Hallegraeff, D. M. Anderson and A. D. Cembella [eds.], Manual on harmful marine microalgae, IOC manuals and guides. UNESCO.

Reguera, B., J. Franco, and P. Fernandez. 1993. Datos preliminares sobre el crecimiento y la produccion de toxinas PSP en cultivos de dos cepas de *Alexandrium lusitanicum*, p. 97-99, III Reunion Iberica sobre Fitoplancton Toxico y Biotoxinas.

Silva, E. S. 1962. Some observations on marine dinoflagellate cultures. I. *Prorocentrum micans* Ehr., *Gymnodinium spinifera* (Clap. and Lach.) dies., *Gonyaulax tamarensis* Leb., and *Peridinium trochoideum* (Stein) Lemm. Notas e estudos do Inst Biol Mar **26:** 1-21.

Silva, E. S., and I. Sousa. 1981. Experimental work on the dinoflagellate toxin production. Separata dos Arquivos do INSA **VI:** 381-387.

Uribe, P., and R. T. Espejo. 2003. Effect of Associated Bacteria on the Growth and Toxicity of *Alexandrium catenella*. Applied and Environmental Microbiology **69:** 659-662.

Utermöhl, H. 1958. Neue Wege in der quantitativen Erfassung des Planktons (mit besonderer Berücksichtigung des Ultraplanktons. Verh. int. Ver. theor. angew. Limnol. **5:** 567-596.

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16. Abstract (Limit: 200 words)
Many species in the dinoflagellate genus *Alexandrium* have been shown to produce saxitoxins, the ethological agent of paralytic shellfish poisoning (PSP). The first case of a toxic *Alexandrium* that has completely lost the ability to produce saxitoxins is reported here. The loss of toxicity was accompanied by a reduction in growth capability. A subculture of this isolate maintains the ability to produce toxins and to grow at rates previously characteristic of this strain. The differences in the two isolates were demonstrated to be a property of the dinoflagellate and were not dependent on the different bacterial symbionts of each culture.

The pair of subcultures is a novel experimental system to study functional genomics of dinoflagellates related to toxin production and growth. At the metabolome level, compounds were identified that were unique to the non-toxic isolate. Their emergence may be correlated to a disruption of the biosynthetic pathway for PSP toxins, as the compounds share some characteristics with saxitoxins. Difference gel electrophoresis (DIGE) identified proteins differentially expressed between the two subcultures. Proteins down-regulated in the non-toxic subculture are enzymes from the Calvin cycle, which may explain the limited growth of the isolate. Other differentially expressed proteins identified may relate to the loss of toxicity, but their identity remains unresolved.

17. Document Analysis **a. Descriptors**
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